# *Mesophilic fermentative hydrogen production from biomass*

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**A submission presented in partial fulfilment**
**of the requirements of the University of Glamorgan/Prifysgol Morgannwg**
**for the degree of Doctor of Philosophy**

**May 2005**

R11

## *Certificate of Research*

*This is to certify that, except where specific reference is made, the work described in this thesis is the result of the candidate. Neither this thesis, nor any part of it, has been presented, or is currently submitted, in candidature for any degree at any other University.*

*Signed* .....
*Candidate*

*Signed* .....
*Director of Studies*

*Date* 11-12-05 .....

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**List of Abbreviations**

| | |
|---|---|
| CSTR | continuously stirred tank reactor |
| FID | flame ionisation detector |
| GC | gas chromatograph |
| HPLC | high performance liquid chromatography |
| HRT | hydraulic retention time |
| IGER | Institute of Grassland and Environmental Research |
| NIST | National Institute of Standards and Technology |
| SS | suspended solids |
| TCD | thermal conductivity detector |
| TS | total solids |
| VFA | volatile fatty acids |
| VS | volatile solids |
| VSS | volatile suspended solids |

**Acknowledgements**

I would like to thank the UK EPSRC for funding through Grant number GR/R22520/01, the Institute of Grassland and Environmental Research (IGER, Aberystwyth) for advice and supply of grass, Cog Moors sewage works (Cardiff) for supply of the anaerobic digester sludge and the staff at the University of Glamorgan, particularly Alvine Jones, Lesley Farley and the School of Technology workshops for all their help and advice. I wish to thank my supervisors for giving me the opportunity to do this research and for their guidance and optimism, and most of all Freda Hawkes for her invaluable support and enthusiasm.

**Abstract**

Hydrogen is considered a possible alternative to fossil fuels. Hydrogen can be produced through dark fermentation with 1 mol hexose yielding a maximum of 4 mol hydrogen in association with acetate production, and 2 mol hydrogen in association with butyrate production. However, an economically and technically feasible process is yet to be established. So far research into fermentative hydrogen production has focused on pure and soluble carbohydrates, particularly glucose. To reduce substrate costs, use of more complex low-cost co- and waste products of the food industry or biomass crops which have undergone minimum pre-treatment would be desirable. Also, whilst much research to date has focused on use of pure bacterial strains, an easily obtainable mixed microflora would be preferable to avoid costs of substrate sterilisation.

Therefore this research project focused on fermentative hydrogen production from three abundant (in the UK) low cost substrates, namely a wheat starch co-product, sugarbeet and perennial ryegrass. Anaerobic digester sludge obtained from the local sewage works was used as inoculum in a continuously stirred tank reactor. Production of hydrogen and other fermentation products was measured to gain information about the main metabolic pathways used. To lower hydrogen partial pressure the reactor was sparged with nitrogen and the effect on hydrogen production observed.

It was demonstrated that stable fermentative hydrogen production from the wheat starch co-product and sugarbeet water extract was possible in continuous operation. Hydrogen production from grass extract was demonstrated in batch mode. Sparging with nitrogen significantly increased hydrogen yields, by 46% for the wheat starch co-product, by 67% for sugarbeet water extract, and by 184% for ryegrass extract. Maximum yields achieved were 1.9 mol hydrogen per mol hexose converted for 16 days on starch, 1.7 mol per mol hexose converted for 5 days on sugarbeet water extract and 0.8 mol hydrogen per mol hexose converted in batch from grass extract. Therefore up to 48% of the maximum theoretical hydrogen yield was produced. Various factors were identified as preventing higher hydrogen yields. Hydrogen production was more closely related to butyrate than acetate concentration. Also, lactate, ethanol and propionate, which are products of carbohydrate fermenting metabolic pathways that do not produce hydrogen, were detected, as were signs of hydrogen consuming homoacetogenesis in continuous operation.

# 1 Introduction

## *1.1 Overview*

Increasing evidence that the Earth's climate has changed significantly due to industrial and domestic green house gas emissions (IPCC 2001) together with the increasing awareness that fossil fuels are a limited resource, largely controlled by politically troubled countries, have initiated the international search for alternative, carbon neutral sources of energy. The UK has a legally binding commitment under the Kyoto protocol to reduce greenhouse gas emissions by 12.5% by 2008-2012 against the 1990 levels and a domestic goal of reducing carbon dioxide emissions by 20% by 2010.

Road transport is responsible for 22% of the UK greenhouse gas emissions. Hydrogen, an entirely carbon-free fuel with a high heating value (lower heating value of 241.8 kJ $mol^{-1}$), is considered a feasible alternative to fossil fuels, with the technology for hydrogen as a transport fuel already well established (for example in the BMW 7 series, the NEBUS by Daimler-Benz and the hydrogen filling station at Munich airport). Currently in the UK 95% of hydrogen is produced by the chemical industry through steam methane reforming at a cost of £4/GJ to £7/GJ, depending on natural gas price (Maddy *et al.* 2003). To reduce carbon dioxide emissions, hydrogen must be produced by a carbon neutral process. Hydrogen production costs of as little as £7/GJ from wind powered electrolysis and £5/GJ from biomass pyrolysis on an industrial scale have been reported (Maddy *et al.* 2003). This compares to a petrol pump price of approximately £0.80 per litre in autumn 2004. Assuming 70% of this is tax (AA 2005), untaxed petrol costs £0.24 per litre. Assuming a combustion energy of 34.5 MJ $l^{-1}$ for petrol (Australian Institute of Energy 2005), this gives a petrol price of £7/GJ. Although this is the untaxed consumer price and not the production costs, it indicates that the costs of renewably produced hydrogen are already within the same magnitude as fossil fuel prices.

Fermentative hydrogen production is currently still in the research stage but, as far as technology is concerned, would only require small changes from anaerobic digestion, which is already well established on an industrial scale. In anaerobic digestion a wide range of organic wastes is stepwise fermented by a mixed anaerobic microbial culture to produce a biogas consisting of 25 to 45% $CO_2$ and 55 to 75% $CH_4$ as shown in Figure 1-1 (Reith *et al.* 2003).

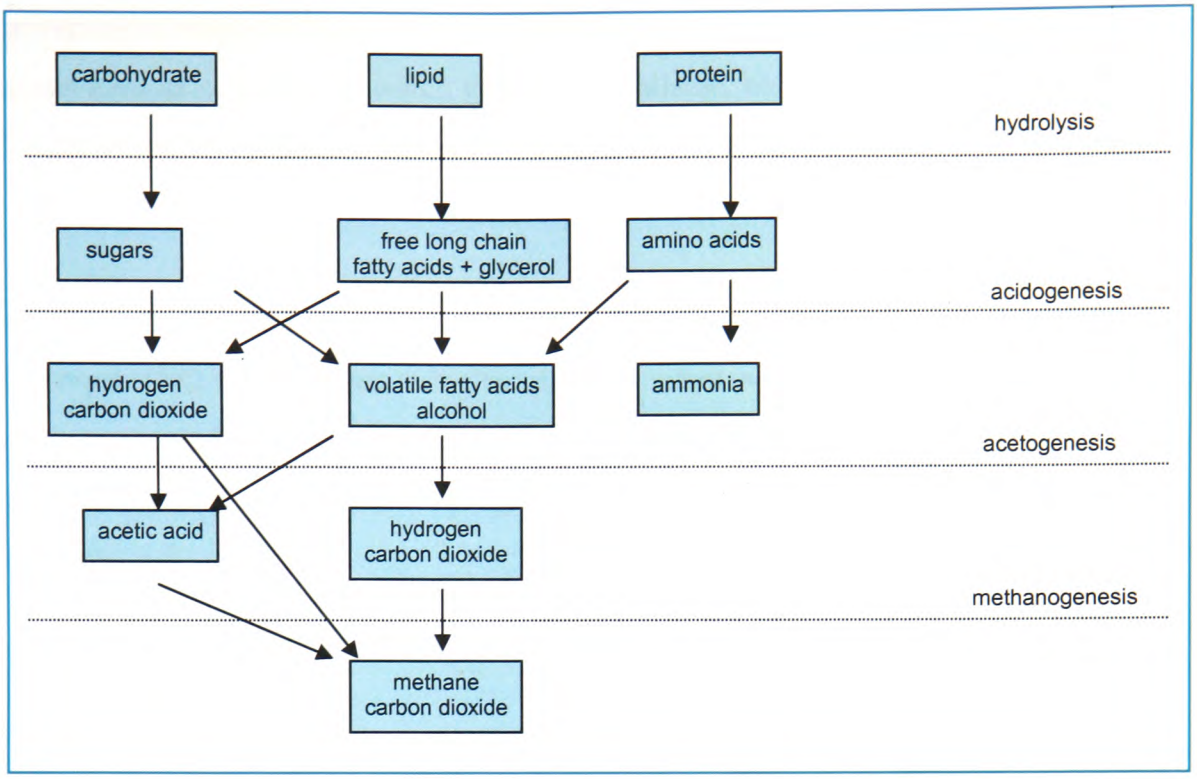



***Figure 1-1. Schematic of organic waste decomposition in anaerobic digestion***

Hydrogen was found to be a product of the acidogenic and obligate hydrogen producing acetogenic phases in this process, and is converted to methane or acetate by hydrogen consumers. In theory the hydrogen consuming homoacetogenic and methanogenic phases can be suppressed through changes in operating parameters of the fermentation process, resulting in production of a biogas consisting entirely of hydrogen and carbon dioxide. This has been shown in foundational work by Cohen and Zoetemeyer (Cohen *et al.* 1979; Zoetemeyer *et al.* 1982c), who developed a 2 stage anaerobic digestion process, separating the acidogenic, hydrogen producing stage physically from the methanogenic stage. In comparison to anaerobic digestion, which is based on symbiosis of several trophic groups, hydrogen production requires exclusion of hydrogen consuming organisms as well as exclusion of competing metabolic groups, which utilise the same substrate but do not produce hydrogen.

Fermentative hydrogen production has several desirable attributes. The substrates used could be organic waste products, as used for anaerobic digestion, and also energy crops, as this would help diversification of agriculture. Conventional agriculture in the UK has become less and less economically viable in recent years, and farmers are increasingly

looking to diversify their business. Amidst other new directions in farming the UK government supports the production of energy crops. Currently this is focused on woody crops such as coppice, for which grants are available, but non-woody energy crops can be grown on set-aside land or will alternatively be subsidised under the new Common Agricultural Policy (DEFRA 2005).

Compared to the methane (55.5 MJ $kg^{-1}$) produced in conventional anaerobic digestion, hydrogen (142 MJ $kg^{-1}$) has a higher energy content per unit mass. Burning hydrogen causes zero carbon emissions. As well as considering biohydrogen production as a sole process of substrate conversion, it could be integrated into a holistic system as sketched in Figure 1-2.

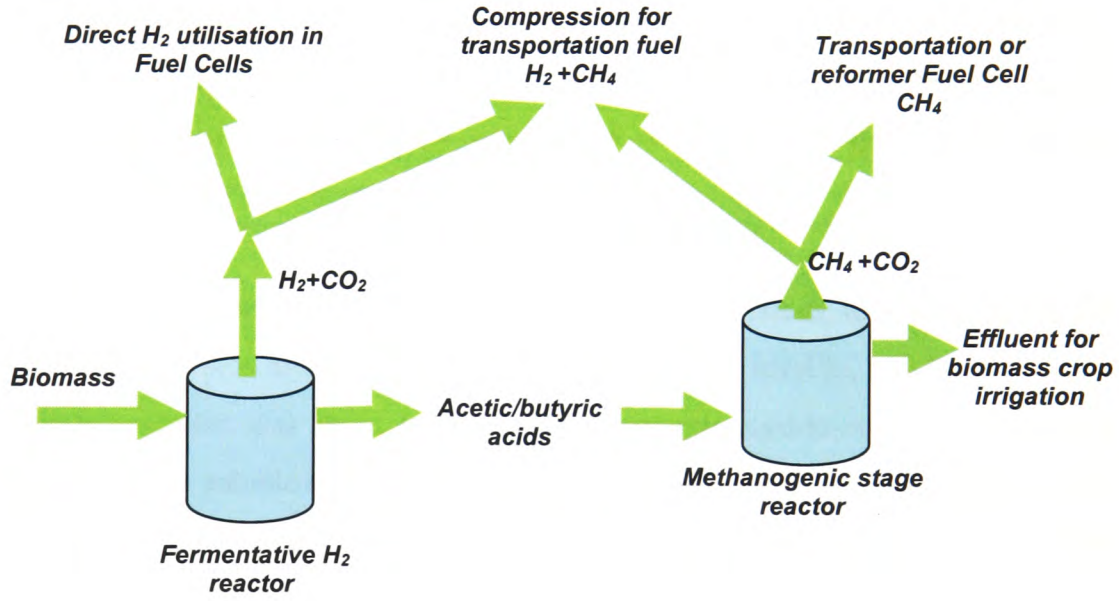



***Figure 1-2. Integration of fermentative hydrogen production into a holistic system.***

The effluent from the hydrogen producing reactor could proceed to an anaerobic digester, in which it would be partly converted to $CO_2/CH_4$ biogas. The effluent of this second reactor would contain the majority of the nitrogen, phosphate and potassium entering the reactor and could be used for crop irrigation in a traceable system.

Compared to most other methods of hydrogen production such as biomass pyrolysis, which require a large scale project to be economically viable, fermentative hydrogen production could operate like anaerobic digestion on a small scale and would therefore

be particularly suitable for remote rural areas, where biomass crops could be processed on-site.

Before this project commenced in 2001, research into fermentative hydrogen production had mainly focused on simple carbohydrates, such as glucose (Mizuno *et al.* 2000a) and sucrose (Fang and Liu 2001), since these are most easily fermented. Research into hydrogen production from polysaccharides focused on batch experiments (Lay and Noike 1999) and use of pure cultures (Yokoi *et al.* 1998b). From an economic point of view, complex carbohydrate substrates, such as low cost waste products and biomass crops, are more desirable substrates than higher value, highly processed and purified products such as sucrose, for which there is already a high demand for other purposes. Use of mixed cultures would also be more desirable, to cut costs involved in the substrate sterilisation required for operation on pure cultures. A continuous process would provide a continuous energy supply and diminish interruptions in hydrogen production caused by regular start-up.

Some information on continuous hydrogen production from mixed cultures and complex substrate was available, but these substrates were soluble, and the duration of continuous operation was short. Lay (2000) for example reports continuous hydrogen production from a soluble starch in experiments lasting 4 days. Data was only collected at one point in time, at the end of each experiment. Similarly, Taguchi *et al.* (1996) reports continuous hydrogen production from cellulose. The cellulose was enzymatically hydrolysed beforehand, and the continuous operation lasted around 4 days. Information on batch experiments from mixed culture and insoluble substrates was also available, but actual hydrogen yields per mol hexose or unit weight substrate converted were not reported, for example by Okamoto *et al.* (2000), who investigated hydrogen production by a mixed culture from fractions of municipal solid waste. More information was required for optimisation of these processes, and no information was yet available on continuous hydrogen production with mixed cultures from particulate substrates.

## *1.2 Aims of the research*

- **To investigate batch and continuous hydrogen production from two carbohydrate rich agricultural crops and a low cost particulate wheat-starch co-product by a mixed microbial culture obtained from the anaerobic digester of the local municipal sewage plant**

- **To optimise hydrogen production through identification of constraints to hydrogen production and through investigation of the effect of inoculum pre-treatment and changes of operational parameters on these constraints and hydrogen yields.**

## *1.3 Microbiology and biochemistry of dark fermentative hydrogen production and consumption*

A wide range of microorganisms are able to fermentatively produce hydrogen from carbohydrates, including obligate anaerobes (e.g. clostridia, ruminococci and archaea), facultative anaerobes (e.g. *Escherichia coli* and *Enterobacter aerogenes*) and some aerobes in anoxic conditions (e.g. *Alcaligenes eutrophus* and *Bacillus licheniformis*) (Claassen *et al.* 1999). To date research into fermentative hydrogen production focused mainly on clostridia and *Enterobacteriaceae*. Hydrogen consumers which must be excluded are methanogens and homoacetogens (a wide range of anaerobes including some clostridia). Metabolic groups of importance to dark fermentative hydrogen production (i.e. groups which produce or consume hydrogen, or compete with hydrogen producers for substrate) will be discussed in this section.

### *1.3.1 Clostridia*

Rogers and Gottschalk (1993) describe clostridia as "anaerobic [endo]spore-forming bacteria that have a Gram-positive type of cell wall and are unable to carry out a dissimilatory sulphate reduction". Clostridia are only known to grow in anerobic conditions, but range from strict anaerobes, such as *Clostridium pasteurianum* and *Clostridium kluyveri* (Schlegel 1993) to almost aerotolerant species such as *Clostridium histolyticum*, *Clostridium acetobutylicum* (Schlegel 1993) or *Clostridium magnum*, which was found to consume trace levels of oxygen (up to 2%), enabling it to survive in

a wider range of environments (Drake and Kuesel 2003). The genus *Clostridium* comprises thermo-, meso- and psychrophilic species and can degrade various organic polymers including starch, cellulose, hemicellulose and protein. In clostridia molecular hydrogen is produced during the anaerobic oxidation of pyruvate, which is produced via the fructose-1,6-bisphosphate pathway from hexose or via the pentose phosphate pathway from pentose (Mitchell 2001). Most clostridia species can ferment substrate to a range of end products, which can be selected for by variation of the growth conditions.

Some peptolytic clostridia are pathogens that cause wound infections (gas gangrene from *Clostridium histolyticum* and *Clostridium septicum* and tetanus from *Clostridium tetani*) and food poisoning (botulism by *Clostridium botulinum*) (Schlegel 1993).

The clostridia are the most commonly found hydrogen-producing genus in anaerobic digester sludge (Cohen *et al.* 1985, Sung *et al.* 2002, Fang, Zhang *et al.* 2002, Lay *et al.* 1999). They are also common in soil (Montoya *et al.* 2000), lake sediments, rotting plant material, rumen and intestinal contents (Brock *et al.* 1994), leguminous roots and cracked cereals (Biebl 1999). Pure strains of clostridia have commercially been used for acetone and butanol production since World War I (acetone was needed for manufacturing of munitions; Jones and Kreis 1995). The process was patented for a strain of *Clostridium acetobutylicum* by Chaim Weizmann in the UK in 1915 (Biebl 1999). Due to development of cheaper chemical synthesis of these solvents from petroleum products in the 1950's, in the West this method was since then only of interest during times of petrol crisis (Brock *et al.* 1994). However, it was still commercially in use in China 10 years ago (Jones and Kreis 1995) and may become economically interesting again when petroleum supplies become sparser.

Clostridial properties and metabolisms of interest to hydrogen production will be discussed in the following sections. An overview of metabolic pathways is given in Figure 1-3 (White 2000).

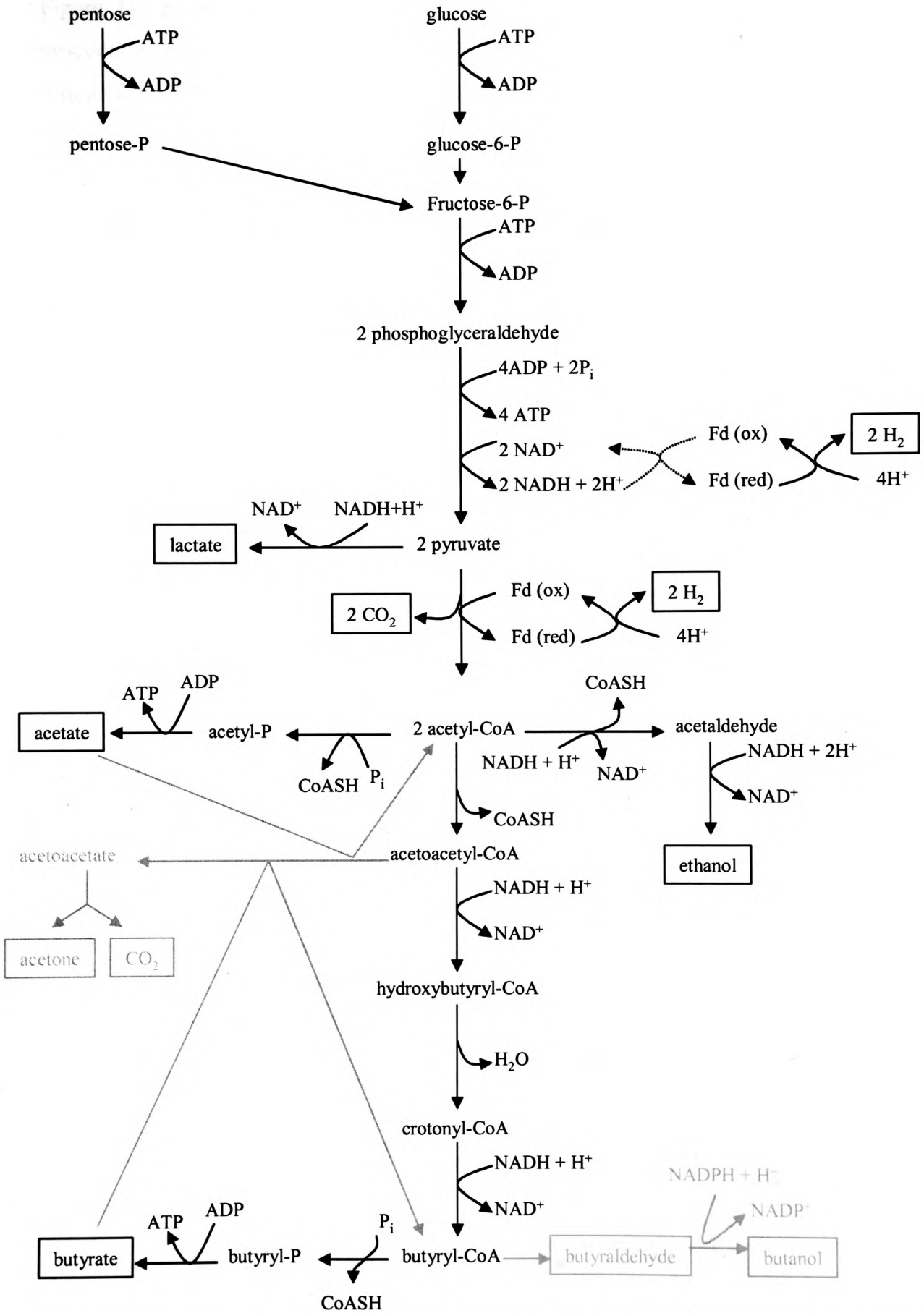



***Figure 1-3. Overview of metabolic pathways of clostridia (White 2000). Pathways of acetone and butanol production drawn in grey.***

Figure 1-3 shows that molecular hydrogen can be produced in two places: in the production of pyruvate and during oxidation of pyruvate to acetyl-CoA. During pyruvate production $NAD^+$ is reduced to NADH + $H^+$, which can be reoxidised through reduction of ferredoxin by NADH-ferredoxin oxidoreductase. This reaction however is endergonic at standard conditions ($\Delta G^{o'} = +18.8$ kJ $mol^{-1}$) and can only proceed at hydrogen partial pressure below $10^{-4}$ atm (Fenchel and Finlay 1995). It is therefore highly unlikely that this reaction would occur in an acidogenic reactor. The ferredoxin reduced in this step as well as the ferredoxin reduced directly during the oxidation of pyruvate to acetyl-CoA can be reoxidised through production of molecular hydrogen by hydrogenase. Instead of production of molecular hydrogen however, ferredoxin can be reoxidised through reduction of $NADP^+$ or $NAD^+$, and these can be reoxidised in the production of reduced endproducts, as for example in the production of lactate, ethanol or butyrate in Figure 1-3. From stoichometric considerations, production of acetate, which does not give opportunity for oxidation of NADPH or NADH other than through production of molecular hydrogen, must therefore be maximised for maximum hydrogen production.

#### 1.3.1.1 Sporulation and germination

In adverse conditions sporulation is an important natural survival mechanism for clostridia (Woods and Jones 1986), but little seems to be known about what exactly these adverse conditions are.

Two aspects of sporulation are of interest for hydrogen production:

- If hydrogen producers in a complex inoculum have formed spores, then these can be selected for by subjecting the inoculum to conditions that kill vegetative cells but not spores.
- During continuous operation sporulation has to be avoided, since it will result in washout of clostridia and will allow non-spore formers such as Selenomonas (see section 1.3.4) to take over in a mixed culture (Cohen *et al.* 1985).

It has been shown that exposure to oxygen triggers sporulation in some species, for example *Clostridium beijerinckii* (Ross *et al.* 1990). Starvation, either of carbohydrate substrate or nutrients, is also assumed to be cause for sporulation (Woods and Jones 1986). Whether sporulation is initiated depends on the length of starvation, the substrate

type and concentration (Woods and Jones 1986) and the species (Ross *et al.* 1990). Ross *et al.* (1990) report from experiments with pure continuous cultures of *Clostridium* strain C7 and *Clostridium beijerinckii*, that the two strains have very different survival mechanisms and thus sporulate in very different conditions. They found that strain *C7* was significantly more tolerant to nutrient stress and particularly to trace concentrations of oxygen than *Clostridium beijerinckii*, which sporulated as soon as trace amounts of air leaked into the reactor.

Overall there seems to be little knowledge about trigger values for sporulation for a wide range of clostridia, and general information is contradictory and confusing. Woods and Jones (1986) for example argue that there is little evidence that spore formation in clostridia is caused by starvation, since an exogenous carbon and energy source is required throughout the sporulation process. Gehin *et al.* (1995) on the other hand state that during the mid-exponential growth phase an exogenous carbon source is not necessarily required, since release of intracellular reserve material (starvation and stress proteins) through the autolysis of a large proportion of vegetative cells enables the cells to sporulate in absence of external carbon. They observed in batch studies with *Clostridium cellulolyticum* on cellobiose, that carbon starvation of cells in the mid-exponential growth phase gave rise to degradation of 20% endogenous protein within 5 hours, followed by sporulation.

Bacterial endospores are described as the most resistant living structure known and have been shown to survive thousands of years (Atrih and Foster 2002), being resistant to adversities such as UV light, solvents, heat (Atrih and Foster 2002), enzyme action, desiccation (Leuschner and Lillford 1999) and pasteurisation (Schlegel 1993). The resistance to chemical and enzymatic attacks is attributed to the multilayered spore coat, which serves as a penetration barrier. Heat resistance is increased by the dehydrated state of the spore core (Atrih and Foster 2002). The spores have no metabolism, but the spore core contains all the necessary metabolic components of the cell plus DNA (Atrih and Foster 2002), enabling the spore to germinate and form a new vegetative cell at reinstatement of more favourable conditions.

Germination has been induced in experiments by amino acids, sugars, ribosides, enzymes and hydrostatic pressure, but often a combination of germinants rather than a

single compound/factor was required (Johnstone 1994). *Clostridium botulinum* spores for example, which are well investigated for food safety reasons, have been shown to germinate optimally (>90% germination) when ≥ 5 mmol $l^{-1}$ L-alanine and ≥ 25 mmol $l^{-1}$ L-lactate were added in experiments testing a range of germinants (Plowman and Peck 2002). However, the compounds triggering germination seem to be species specific. Broussolle *et al.* (2002) for example report that germination of *Clostridium botulinum* was triggered by a combination of L-alanine, L-lactate and $NaHCO_3$, but they also mention that this was not the case for *Clostridium pasteurianum* and *Clostridium actobutylicum* (no details given).

The germinants activate a germination receptor, which causes the spore to start germination, to which the spores are committed from a very early stage, even if the germinant is then removed (Johnstone 1994). Germination of clostridial spores is generally less susceptible to oxygen inhibition than subsequent growth (Plowman and Peck 2002). The germination receptor can be altered by heat shock so that it is more responsive to the presence of germinants (Johnstone 1994), as for example shown by Plowman and Peck (2002) for *Clostridium botulinum* when heat-treated at 60°C for 15 min.

Within one minute from addition of germinants spores have been observed to loose their heat-resistance. This is associated with release of dipicolinic acid and followed by hydrolysis of peptidoglycan in the spore cortex and onset of spore metabolism (Johnstone 1994), developing a new vegetative cell.

#### 1.3.1.2 Butyrate-acetate metabolism

The butyrate-acetate metabolism is the main known hydrogen producing metabolism, as described in Equation 1 and Equation 2 (Biebl 1999; Vavilin *et al.* 1995). 50% of all clostridial species isolated by 1989 (Ljungdahl *et al.* 1989) were reported to metabolise carbohydrates in this way during exponential growth.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2\,CH_3COOH + 2CO_2 + 4H_2 \qquad \textit{Equation 1}$$

$$C_6H_{12}O_6 \rightarrow CH_3\,CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad \textit{Equation 2}$$

From Equation 1 and Equation 2 it can be concluded that the theoretical maximum hydrogen yield for acetate production is 4 mol per mol hexose, and 2 mol per mol

hexose for butyrate production, which would make acetate production more desirable for hydrogen production (Mosey 1983). In practice, only mixed acetate-butyrate fermentation has been observed in mixed cultures to date, as for example in Equation 3 (Ljungdahl *et al.* 1989).

$$4\ C_6H_{12}O_6 + 2H_2O \rightarrow 3\ CH_3CH_2CH_2COOH + 2\ CH_3COOH + 8\ CO_2 + 10\ H_2 \qquad \textit{Equation 3}$$

From the perspective of energy yields for the microorganism, acetate production should be favoured over butyrate production, since each mol glucose yields 4 mol ATP when acetate is produced in comparison to only 3 mol ATP when butyrate is produced. The reason for mixed fermentation is probably the problem of $NAD^+$ regeneration. During acetate production NADH needs to be regenerated by the enzyme NADH-ferredoxin oxidoreductase in a reaction that is endergonic at standard conditions ($\Delta G^{o'} = +18.8$ kJ $mol^{-1}$) and can only proceed at hydrogen partial pressure below $10^{-4}$ atm (Fenchel and Finlay 1995). If this reaction is inhibited, the NADH can be re-oxidised by change of metabolism to other, more reduced, end products. During butyrate metabolism for example, NADH reduced during pyruvate formation is re-oxidised for butyryl-CoA formation. It is thought that for clostridia mixed butyrate/acetate fermentation provides optimum balance between maximising energy yield and recycling NADH (Fenchel and Finlay 1995).

#### 1.3.1.3 Acetone-butanol production

At the end of exponential growth the metabolism of some clostridial strains changes from acid to acetone-butanol producing in certain environmental conditions. Known solvent forming species are for example the mesophilic *Clostridium acetobutylicum*, *Clostridium aurantibutylicum*, *Clostridium tetanomorphum* and *Clostridium beijerinckii* (Mitchell 1998) and the thermophilic *Clostridium thermosaccharolyticum*, *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum* (Ljungdahl *et al.* 1989).

For the industrial solvent production introduced in section 1.3.1 molasses and starchy substrates such as maize meal and potato mash were fermented by pure strains of *Clostridium acetobutylicum* in batches at feed concentrations of 8 to 10%. Maize meal and potato mash could be fermented without addition of nutrients, but molasses had to be supplemented with nitrogen and in some cases with phosphate (Biebl 1999). The

spores of the pure strain, most commonly a strain of *Clostridium acetobutylicum*, were activated by heat-shock (e.g. 2 min at 100°C) and then scaled up in several stages (Biebl 1999). Medium and reactor were sterilised by steam injection. The fermenters were not stirred, but temperature controlled at 30 to 37°C (Duerre 1998). The pH of the medium was initially adjusted to 6.0, then allowed to decrease naturally (Jones and Woods 1986). Maximum obtainable solvent concentrations were around 2% from molasses (Jones and Woods 1986). The fermentation was complete after 30 to 60 hours, and followed by distillation and purification of solvents in fractionating columns (Biebl 1999).

Despite the use of this process on industrial scale, the trigger mechanisms for the switch from acid to solvent production are still poorly understood. Accumulation of undissociated butyric acid in the cell appears to be a key factor, whilst a relation of the concentration of the undissociated acetic acid and solvent formation is not known (Mitchell 1998).

Undissociated acids can pass across the cell membrane, allowing protons to enter the cell. If the concentration of these acids becomes too high, the pH gradient across the membrane collapses, which inhibits the bacterial metabolism. For *Clostridium acetobutylicum* trigger concentrations of undissociated butyric acid of 1500 to 1900 mg $l^{-1}$ (17 to 22 mmol $l^{-1}$) are reported (Jones and Woods 1986), but this value was found to vary with species and catabolic rate. The shift to solvent production may be a mechanism for the cell to detoxify from acid end products, since a large part of these acids are re-assimilated during solvent production. Jones and Woods (1986) for example state in their review that 55 and 85 % of $^{14}C$ from acetate and butyrate respectively were recovered in the produced butanol.

The concentration of undissociated acids in the cells depends on total acid concentration as well as pH. The $pK_a$ of butyric and acetic acid is 4.81 and 4.78 respectively. This means for example that only 6% of total butyric acid is undissociated at pH 6.0, but 66% at pH 4.5 (Jones and Woods 1986). Culture pH trigger values from acid to solvent production of 4.3 to 5.5 for different strains of *Clostridium acetobutylicum* have been reported (Rogers and Gottschalk 1993; Biebl 1999; Bahl and Gottschalk 1984), but solvent production has also been reported at pH as high as 7.0, when high

concentrations of acetate and butyrate were added (no details given; Jones and Woods 1986). In continuous operation, solvent production was most successful at a long hydraulic retention time (improving steadily in the range of 8 to 50h HRT), low pH of 4.3 and phosphate or sulphate limitation (Bahl and Gottschalk 1984).

There are several studies indicating that nutrient limitation, such as phosphate, sulphate (Bahl and Gottschalk 1984; Duerre 1998) or nitrogen limitation (Monot and Engasser 1983) is required for solvent production. However, Jones and Woods (1986) argue that in the industrial solvent production process nutrients were usually in excess, and that there are several studies reporting continuous solvent production in which all nutrients were in excess.

Although butanol production does not involve hydrogen consumption (Equation 4), and acetone production is even associated with hydrogen production (Equation 5), solvent production is not desirable for continuous hydrogen production, as it is associated with the end of exponential growth and often with sporulation (Biebl 1999).

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2CH_2OH + 2CO_2 + H_2O \qquad \textit{Equation 4}$$

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3COCH_3 + 3CO_2 + 4\,H_2 \qquad \textit{Equation 5}$$

To prevent solvent production it would therefore be necessary to keep the hydraulic retention time as short as clostridial growth allows, and to strike a balance between relatively low feed concentrations (to keep the total acid concentrations limited) and a not too low pH (to keep the percentage of undissociated acid low).

#### 1.3.1.4 Lactate and ethanol production

Lactate and ethanol are both reduced end-products of relatively simple metabolic pathways. The energy yield for clostridia is comparatively low from lactate and ethanol production; whilst each mol glucose yields 4 or 3 mol ATP when acetate or butyrate are produced respectively, only 2 mol ATP are gained when lactate or ethanol are produced.

Lactate can be directly produced through reduction of pyruvate by some clostridia, providing a simple mechanism of NADH oxidation, when protons and electrons can not be disposed of through generation of molecular hydrogen (Jones and Woods 1986), for example at high hydrogen partial pressure or iron deficiency in cells (Dabrock *et al.* 1992). Payot *et al.* (1999) for example conclude from batch experiments with *Clostridium cellulolyticum* on cellobiose at pH 7.2 and 34°C, where lactate was the main product after an initial phase of dominant acetate production, that lactate production was a way for the organism to cope with excess NADH produced in association with acetate.

Although lactate production does not involve hydrogen production or consumption (Equation 6; Tanisho and Ishiwata 1994), it is of interest as it competes for substrate with hydrogen production.

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OCOOH \qquad \textit{Equation 6}$$

Lactate production has been observed particularly at pH above 5.0, by *Clostridium acetobutylicum* under sulphate limitation (but not at phosphate limitation, Bahl and Gottschalk 1984), *Clostridium butyricum* (Heyndrickx *et al.* 1990), and *Clostridium thermolacticum* (at 60 to 65°C; Rogers and Gottschalk 1993). It has also been reported from *Clostridium cellobioparum* and *Clostridium thermocellum* (Brock *et al.* 1994), *Clostridium fallax* (Buchanan and Gibbons 1975), *Clostridium cellulolyticum* on cellulose, glucose and cellobiose (Giallo *et al.* 1985) and *Clostridium barkeri* (Ljungdahl *et al.* 1989), but in these publications preferred environmental conditions were not given.

As for lactate production, no hydrogen is produced or consumed during ethanol production from hexose (Equation 7).

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \qquad \textit{Equation 7}$$

For ethanol production formation of acetyl CoA is required but, unlike acetate production, it then involves re-oxidation of NADH. Ethanol is thus also likely to be produced when excess NADH from acetate production can not be regenerated through

production of molecular hydrogen, for example at hydrogen partial pressure above $10^{-4}$ atm. Only few clostridial strains are known to have ethanol as a significant endproduct (Mitchell 1998). Most of these are thermophilic, for example *Clostridium thermocellum*, *Clostridium thermohydrosulfuricum* (Pollach *et al.* 2002) and *Clostridium thermosaccharolyticum*. One known mesophilic ethanol producing organism is *Clostridium saccharolyticum*. Its main metabolic products are acetate, hydrogen and carbon dioxide and it can grow at pH 6.0 to 8.8 and 17 to 43 °C on mono- and disaccharides but not on cellulose or starch (Murray *et al.* 1982). Ethanol as a major product of glucose fermentation with anaerobic sludge inoculum, with or without heat-treatment, has been reported by Oh *et al.* (2004) in batch experiments at pH 6.2 and 7.5. As a minor by-product it has also been reported in pure cultures of *Clostridium butyricum* and *Clostridium pasteurianum* (Heyndrickx *et al.* 1990).

#### 1.3.1.5 Acetogenesis

At very low hydrogen partial pressures a wide range of clostridia can convert various products of carbohydrate fermentation, such as ethanol or propionate, to hydrogen, carbon dioxide and acetate (for example Equation 8 for ethanol and Equation 9 for propionate; Sahm 1984). A hydrogen partial pressure of less than 0.1 Pa for example is required for oxidation of propionic to acetic acid (Sahm 1984). In a natural environment acetogenesis would occur where clostridia live in symbiosis with methanogens, which would keep the hydrogen partial pressure low enough.

$$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2 \qquad (\Delta G_o' = +6.2kJ\ mol^{-1}) \qquad \textit{Equation 8}$$

$$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2 \qquad (\Delta G_o' = +48.1kJ\ mol^{-1}) \qquad \textit{Equation 9}$$

In a hydrogen producing culture optimised to suppress hydrogen consumers, the hydrogen partial pressure is unlikely to be low enough to allow acetogenesis. Clostridia are also able to carry out homoacetogenesis, which will be discussed in section 1.3.3.

### *1.3.2 Facultative anaerobes*

Several facultative anaerobes, particularly in the family *Enterobacteriaceae*, have been shown to produce hydrogen in batch and continuous experiments. *Enterobacteriaceae* are non-spore forming, whilst *Bacillus* species, which can also produce hydrogen, are

endospore forming. Unlike clostridia, facultative anaerobes can respire in aerobic conditions and thus grow in a much wider range of redox potentials. It has also been observed that, unlike in clostridia, growth of *Enterobacter aerogenes* is uninhibited by a gas atmosphere of 100 % $H_2$ (Nakashimada *et al.* 2002).

Similarly to the clostridia, they are able to use a range of metabolic pathways in anaerobic conditions. Fermentations by facultative anaerobes are commonly split into two types: the 2,3-butanediol and the mixed acid fermentation. In the 2,3-butanediol fermentation 2,3-butanediol and acetoin are major products, and more carbon dioxide and ethanol and less acid are produced. Species of *Enterobacter* for example are butanediol fermenters, whilst species of *Escherichia* are mixed acid fermenters (Gregory *et al.* 1978). In both fermentation types, as in clostridial fermentation, hexoses are metabolised to pyruvate via the fructose-bisphosphate pathway and pentoses via the pentose-phosphate pathway (Schlegel 1993).

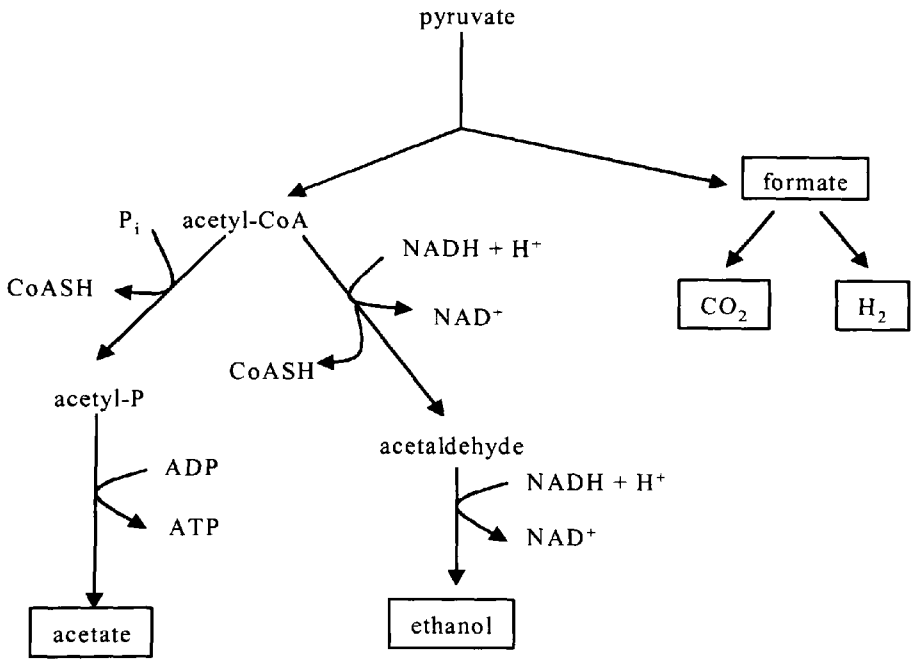



***Figure 1-4. Hydrogen production from pyruvate by facultative anaerobes.***

As Figure 1-4 shows, the metabolism of enteric bacteria then diverts from that of clostridia in that pyruvate is converted by pyruvate-formate lyase to acetyl-CoA and formate rather than oxidised to acetyl-CoA and carbon dioxide. However, as the formate is then broken down to hydrogen and carbon dioxide, the formate producing pathway

yields the same amounts of hydrogen as the clostridial pathway. The distribution of endproducts is determined by a balance of maximum ATP yield (through acetate production) and the need for regeneration of NADH (ethanol production for example utilises 4 [H], lactate 2 [H] and acetate 0 [H]). Production of ethanol and butanediol is most likely favoured at low pH to reduce further acidification (White 2000). It appears that some but not all enteric strains can produce hydrogen directly by oxidisation of NADH via ferredoxin-NADH oxidoreductase like clostridia (Nakashimada *et al.* 2002), but this pathway is not commonly used. This theory would for example be supported by reports of hydrogen yields of 2.76 mol per mol hexose from *Citrobacter* sp. Y19 (Oh *et al.* 2003) and 3 mol hydrogen per mol hexose from sucrose by *Enterobacter cloacae* (Kumar and Das 2000). These yields exceed the theoretical maximum yield of 2 mol hydrogen produced per mol hexose from formate conversion, which indicates the presence of a second hydrogen producing mechanism. Oh *et al.* (2003) states that yields could be increased by 40 % if the culture was repeatedly purged with argon, lowering the hydrogen partial pressure (but the data was not shown and no details given). This may indicate that one or both of the hydrogen producing mechanisms are limited by hydrogen partial pressure.

### *1.3.3 Homoacetogenesis*

Homoacetogenesis is defined by a metabolic pathway, in which 2 mol carbon dioxide are reduced to form 1 mol acetate via the acetyl-CoA (also named Wood-Ljungdahl) pathway (Drake and Kuesel 2003). For hydrogen production this metabolism is of interest when molecular hydrogen is used as electron donor, as this causes hydrogen consumption as shown in Equation 10.

$$2\,CO_2 + 4\,H_2 \rightarrow CH_3COOH + 2H_2O \qquad \Delta G_o' = -100\ kJ\ mol^{-1} \qquad \textit{Equation 10}$$

It is also reported that some organisms are able to convert 1 mol hexose directly to 3 mol acetate without any by-products as in Equation 11 (Drake and Kuesel 2003; Ljungdahl *et al.* 1989; Mitchell 2001).

$$C_6H_{12}O_6 \rightarrow 3\,CH_3COOH \qquad \textit{Equation 11}$$

However it is not clear if this is a single or a two step reaction as represented by Equation 12 and Equation 13.

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,CH_3COOH + 2\,CO_2 + 8\,H^+ + 8\,e^- \qquad \textit{Equation 12}$$

$$2\,CO_2 + 8\,H^+ + 8\,e^- \rightarrow CH_3COOH + 2\,H_2O \qquad \textit{Equation 13}$$

To date this metabolism is only well investigated for a very small number of bacterial strains, particularly Moorella thermoacetica and Moorella thermoautotrophica (Das and Ljungdahl 2000). Homoacetogenic bacteria in general seem to be rather difficult to account for, as a large number of highly diverse bacterial groups as well as a number of taxonomically unclassified isolates are known to be homoacetogenic. In 2003 19 genera were known to be able to use the homoacetogenic pathway (Drake and Kuesel 2003), including *Acetobacterium, Eubacterium, Ruminococcus, Sporomusa* and *Thermoanaerobacter.* Some clostridial species are known to be homoacetogenic, for example *Clostridium glycolyticum* (Drake et al. 2002) and *Clostridium magnum* (Ljungdahl et al. 1989).

In addition to the genetic, phenotypical and phylogenetical diversity of homoacetogens (Das and Ljungdahl 2000), identification of homoacetogenic strains is further complicated by the fact that most bacterial species which are termed homoacetogens in earlier publications (e.g. Ljungdahl *et al.* 1989) are now known not to be strictly homoacetogenic and are therefore more correctly termed acetogens (Drake and Kuesel 2003). Most acetogens are now considered to be facultative autotrophs since they are known to utilise in addition to carbon dioxide a wide range of substrates including sugars, alcohols, aromatic compounds, carbon monoxide, sulfite, nitrate (Das and Ljungdahl 2000), aldehydes and carboxylic acids (Drake and Kuesel 2003). Several of these pathways do not involve consumption of molecular hydrogen and do not have acetate as end-product (Ljungdahl *et al.* 1989). Furthermore, molecular hydrogen is also known as an end-product (no details given; Drake and Kuesel 2003).

According to Drake and Kuesel (2003) acetogens can switch metabolism depending on growth conditions. *Moorella thermoacetica* for example prefers to reduce nitrate rather than carbon dioxide if available. The occurrence of homoacetogenesis is therefore predominantly determined by availability of electron acceptors and environmental conditions rather than an absolute characteristic of a species (Drake *et al.* 2002).

To maximise hydrogen production in a mixed culture, homoacetogenesis must be suppressed. However, due to the high diversity and flexibility of (homo-)acetogens, very little can be found in the literature on growth conditions which would favour or discourage homoacetogenesis. On the contrary, actogenic bacteria have been found in a wide range of natural environments.

The ability to use various electron acceptors allows the bacteria to grow in a range of fluctuating redox conditions. Drake and Kuesel (2003) report that some hydrogen utilising acetogens, for example *Clostridium magnum*, are not only able to cope with, but even consume trace levels (up to 2%) of oxygen. *Clostridium glycolyticum* was found to tolerate up to 6% oxygen in the headspace of continuously shaken culture tubes (Drake *et al.* 2002). Although most homoacetogens have been found in obligately anoxic habitats, such as anaerobic sediments, sewage sludge and gastrointestinal tracts, due to their oxygen tolerance they have also been isolated from more fluctuating environments such as hypersaline sediments, oxic soils and plant roots.

Mesophilic as well as psychrophilic and thermophilic homoacetogens have been isolated. Although pure cultures of methanogens were able to utilise hydrogen at lower hydrogen partial pressure than homoacetogens, it has been observed in lake sediments that homoacetogens thrived at pH 6.2 and were able to out-compete methanogens for hydrogen (Drake and Kuesel 2003). In a range of natural environments (rice paddy soils, tundra wetlands) it has been observed that low temperature, low pH and carbon limited environments (no details given) favour homoacetogenesis over methanogenesis (Drake and Kuesel 2003).

In summary it must be concluded that there are no temperature or pH ranges known to date which discourage homoacetogenesis. Since the homoacetogenic pathway, which involves hydrogen consumption, will only be used if it is energetically favourable, the

most obvious means of suppressing homoacetogenesis is the reduction of the hydrogen partial pressure.

### *1.3.4 Propionate producers*

In propionic acid type fermentation lactate (Equation 14; Brock *et al.* 1994) or carbohydrate (Equation 15; Gottschalk 1979) are consumed to produce propionate.

$$3\ CH_3CHOHCOOH \rightarrow 2\ CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O \quad \textit{Equation 14}$$

$$1.5\ C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O \quad \textit{Equation 15}$$

There are at least two pathways of propionate production, both of which yield 1 mol acetate and 2 mol propionate from 3 mol lactate or 1.5 mol hexose and do not produce or consume net hydrogen in this product ratio. In one of the two pathways lactate and carbohydrate can be converted to propionate via pyruvate. The 6 mol [H] produced during substrate conversion to 3 mol pyruvate plus the 2 mol [H] produced with each mol acetate are re-utilised in the production of 2 mol propionate. This pathway is used by the genus *Propionibacterium*, the most commonly mentioned propionate producing bacteria (White 2000), which are for example responsible for the unique flavour of Emmentaler cheese (Brock *et al.* 1994). They are also commonly present in rumen and on human skin, but unlike clostridia are not common in soil (Schlegel 1993). Brock *et al.* (1994) states that these bacteria are slow growing (no details given), non-spore forming facultative anaerobes and can therefore possibly be inhibited by short hydraulic retention times (see section 1.6.4) and heat pre-treatment (see section 1.5).

Cohen *et al.* (1985) report that during propionic acid type fermentation in an acidogenic reactor the microbial community consisted mainly of Gram-positive, curved rods, tentatively identified as *Selenomonas* sp, which, like propionibacteria, are not spore-formers and produce propionate via pyruvate (Schlegel 1993).

There are also some propionate producing clostridial species, such as *C propionicum*, *Clostridium novyi* and *Clostridium arcticum*. Of these, *C propionicum* is most extensively studied (Ljungdahl *et al.* 1989) and reported to produce propionate from

lactate only, via a relatively simple pathway not involving pyruvate production, yielding 1 mol ATP per 3 mol lactate converted (White 2000). The hydrogen balance is the same as for the *Propionibacterium*: conversion of 1 mol lactate to acetate yields 4 mol [H], which are re-utilised in conversion of 2 mol lactate to propionate (Equation 14) (White 2000). Since this metabolism does not produce or consume hydrogen and does not involve consumption of hexose, it has no effect on the overall hydrogen yield from hexose in a mixed culture.

It appears that equation (3) of Vavilin *et al.* (1995), in which one mol hexose and 2 mol hydrogen are converted to 2 mol propionate and 2 mol carbon dioxide, as also quoted in Hawkes *et al.* (2002), is a theoretical stoichiometric equation and does not wholly represent any of the propionate producing metabolisms.

### *1.3.5 Methanogens*

Methanogens are a highly specialised group of Archaea, which are essential for the anaerobic digestion process, as they are the dominant hydrogen consumers (Equation 16), as well as the main group breaking down acetate to inorganic end products (Equation 17) (Fenchel and Finlay 1995). Figure 1-1 showed methanogenesis as the final step in the anaerobic degradation process.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad (\Delta G_o' = -136\ kJ\ mol^{-1}) \qquad \textit{Equation 16}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad (\Delta G_o' = -31\ kJ\ mol^{-1}) \qquad \textit{Equation 17}$$

Almost all methanogenic species can rapidly convert hydrogen according to Equation 16 (McInerney and Bryant 1981). Thus for biohydrogen production, the main change to be made from single stage anaerobic digestion is the inhibition of methanogens.

The methanogen group comprises a wide range of species including rods, cocci, plate-shaped and filamentous shapes (Brock *et al.* 1994). They are common in rumen, freshwater swamps and marshes, and anoxic micro-environments such as the centre of soil crumbs (Brock *et al.* 1994). Methane production in natural environments has been observed at 0 to 97°C, and some methanogenic species have particularly adapted to

extreme temperature environments, such as submarine hot vents close to 100°C (Fenchel and Finlay 1995). All of these species however have some common growth characteristics:

- They are strict anaerobes and require a redox potential below –300 mV (McInerney and Bryant 1981).
- In healthy anaerobic digesters as well as in their natural habitats methanogens usually maintain the hydrogen partial pressure at less than $10^{-4}$ atm, which means that hydrogen consumption is in reality less energy efficient than in standard conditions (Equation 16) with a $\Delta G'$ closer to –40 kJ $mol^{-1}$. Therefore both metabolic pathways have very low energy efficiency and consequently all methanogens have a very low growth rate (Fenchel and Finlay 1995).
- The optimal pH range for growth is 6 to 8 (Sahm 1984), although reduced growth was also observed outside this range (Speece 1996).

### *1.4 Hydrogen production from pure cultures*

To date hydrogen production has been reported and quantified from batch and continuous experiments with pure strains of several obligate and facultative anaerobic species of the bacterial groups introduced in section 1.3.

A summary of species used and hydrogen yields achieved is given in Table 1-1, which shows that hydrogen yields from pure cultures range from 15 to 75 % of the theoretical maximum yields (Equation 1). Overall, hydrogen yields achieved from clostridia appear to be higher than those from *Enterobacteriaceae* (Yokoi *et al.* 1998b), although very high yields have been reported for some enteric species, for example 3 mol hydrogen per mol hexose from sucrose by *Enterobacter cloacae* (Kumar and Das 2000). *Enterobacteriaceae* on the other hand have due to their tolerance to oxygen a clear advantage over some clostridial species (Tanisho and Ishiwata 1994). Yokoi *et al.* (1995) showed that a combination of pure cultures *of Clostridium butyricum* and *Enterobacter aerogenes* strain HO-39 for example achieved the best of both. In batch experiments with the two strains the hydrogen yield was increased from 1 mol per mol hexose from a pure culture of *Enterobacter aerogenes* strain HO-39 only, to 2.6 mol per mol hexose for the two strains combined. *As Enterobacter aerogenes* consumed any oxygen and provided strictly anaerobic conditions for *Clostridium butyricum*, this also

made addition of a reducing agent unnecessary, which was essential for hydrogen production from the strain of *Clostridium butyricum* only (Yokoi *et al.* 1998b).

***Table 1-1. Species, substrates and hydrogen yields of pure culture studies***

| Species | substrate | $H_2$ yield [1] | Mode | Reference |
|---|---|---|---|---|
| *Clostridium butyricum* | glucose | 2.2 [2] | Cont. | (Heyndrickx *et al.* 1990) |
| *Clostridium butyricum* | glucose | 2.3 | Cont. | (Kataoka *et al.* 1997) |
| *Clostridium butyricum* | glucose | 1.8 | Cont. | (Van Andel *et al.* 1985) |
| *Clostridium butyricum* | sweet potato starch residue | 2.4 | Batch | (Yokoi *et al.* 2001) |
| *Clostridium pasteurianum* | glucose | 2.4 | Batch | (Brosseau and Zajic 1982b) |
| *Clostridium pasteurianum* | glucose | 2.1 [2] | Cont. | (Heyndrickx *et al.* 1990) |
| *Clostridium* sp. No. 2 | xylose | 2.1 [2] | Cont. | (Taguchi *et al.* 1995) |
| **Enterobacteriaceae** | | | | |
| *Enterobacter aerogenes* | hydrolysed starch | 1.0 | Batch | (Fabiano and Perego 2002) |
| *Enterobacter aerogenes* | molasses | 0.8 [2] | Cont | (Tanisho and Ishiwata 1994) |
| *Enterobacter aerogenes* Strain HO-39 | glucose | ~ 1 | Cont. | (Yokoi *et al.* 1995) |
| *Enterobacter cloacae* | glucose, | 2.2 | Batch | (Kumar and Das 2000) |
| | sucrose, | 3.0 | Batch | |
| | cellobiose | 2.7 | Batch | |
| *Citrobacter intermedius* | glucose | 1.5 | Batch | (Brosseau and Zajic 1982b) |
| *Citrobacter sp. Y19* | glucose | 2.5 [2] | Batch | (Oh *et al.* 2003) |
| *Bacillus licheniformis* | wheat slurries | 0.7 [2] | Batch | (Kalia *et al.* 1994) |

[1] [mol hydrogen per mol hexose added] unless stated otherwise, see [2]

[2] [mol hydrogen per mol hexose converted]

The main advantage of hydrogen production with pure cultures over operation with mixed cultures is that it naturally prevents hydrogen consumption by other species. Therefore process optimisation can focus entirely on maximum growth of the selected strain, whilst in mixed cultures equal emphasis has to be given to prevention of growth of hydrogen consuming or competing non hydrogen producing organisms. Also, strains can be selected for cost of maintenance of their optimum growth conditions. Yokoi *et al.* (1995) for example explain their choice of *Enterobacter aerogenes* strain HO-39 for its ability to grow at the relatively low pH 4.0, which makes the operation of a reactor cheaper, since less alkali is needed for pH control.

However, exclusion of other species does not prevent the selected strain from varying its dominant metabolic pathways. As discussed in section 1.3, clostridia and *Enterobacteriaceae* both have a range of metabolic pathways available, producing more or less hydrogen (acetate/butyrate metabolism, reduced end products) or even consuming hydrogen (homoacetogenesis). Kataoka *et al.* (1997) for example report from experiments with a pure culture of *Clostridium butyricum* strain SC-E1 that the metabolism during continuous operation changed from predominantly acetate/butyrate producing to predominantly formate/lactate producing after around 7 days. This change in dominant metabolism coincided with a decrease in hydrogen yield from over 2 mol per mol hexose to less than 1.5 mol per mol hexose.

The use of pure cultures has also several disadvantages. The main problem is that the medium has to be sterilised. Taguchi *et al.* (1995) for example autoclaved their medium for 15 minutes. This will significantly increase the necessary energy input into the system. I am not aware of any study that analyses if increases in hydrogen yield by pure cultures in comparison to mixed microflora justify this extra energy input. For solvent production sterilisation was obviously cost effective until synthesis from petroleum was developed. However, fermentative solvent production did not need to be energy efficient. Additional costs arise from addition of yeast extract or peptone, which is common practise for pure cultures and has been shown to be essential for some strains, e.g. by Yokoi *et al.* (1998a) for a strain of *Clostridium butyricum*, as well as reducing agents such as L-cysteine, for most clostridial strains. Biebl (1999) further point out that infection with bacteriophage was a serious problem when using pure clostridial cultures for industrial solvent production, which made it necessary to keep spores of a large number of strains so that a different strain could be used if one was infected by a phage.

Also, to achieve higher yields than could be achieved with mixed cultures, the pure strain needs to be carefully selected for each substrate, since many species specialise in certain substrates. Roychowdhury *et al.* (1988) for example conclude from a set of batch studies with pure cultures of *E. coli* or *Citrobacter* spp. and mixed cultures derived from sewage or landfill dust, that hydrogen yields from saccharified cellulose were much lower with the pure cultures than the mixed cultures (values for hydrogen yields not given).

### *1.5 Mixed microflora and pre-treatments*

Pure cultures in sterile conditions may be the most obvious choice to avoid contamination with other microorganisms, but hydrogen production from stable mixed microflora easily obtainable from natural sources able to operate on non-sterile feedstock would be economically and technically more feasible, as it would cut out the cost and complication of sterilising reactor and feed.

Hydrogen production has for example been reported from the following inocula: activated sludge (Cohen *et al.* 1979), sludge compost which was manufactured from aerobic activated sludge by forced aeration (Ueno *et al.* 1995), anaerobic digestion sludge obtained from thermophilic methanogenic fermentation (Ueno *et al.* 1995), fresh horse faeces (Oi *et al.* 1982), potato and soybean soil and compost (Van Ginkel *et al.* 2001).

Analysis of the microbial community in cultures originating from these sources showed that clostridia were commonly the dominant genus in mesophilic conditions. Fang, Zhang *et al.* (2002) for example analysed the microbial species present in a hydrogen producing culture at pH5.5, 36 °C and 6.6h HRT. The inoculum for the experiment was sludge from the secondary sedimentation tank of a wastewater treatment plant, the substrate was glucose. They found that 64.4% of all clones present were *Clostridiaceae*, with 43.8% being most closely related to *Clostridium cellulosi*, 12.5% most closely related to *Clostridium acetobutylicum* and 8.3% most closely related to *Clostridium tyrobutyricum*. 18.8% of all clones were affiliated with *Enterobacteriaceae*, and 3.1% with *Streptococcus bovis*.

Sung *et al.* (2002) identified the dominant microbial groups of a mesophilic hydrogen producing culture on sucrose, derived from heat-treated anaerobic digester sludge, during the first 15 days of continuous operation as *Clostridium butyricum* and one or more of the species *Clostridium beijerinckii*, *Clostridium botulinum*, *Clostridium putrificum* and *Clostridium sporogenes*. However, although the hydrogen production remained stable, the microbial community changed after 15 to 22 days. The culture was then found to be dominated by *Bacillus laveolaticus*, also a spore former. It is not clear if the bacillus dominated culture was imported gradually for example with the substrate, or if it originated from the inoculum.

When Ueno *et al.* (2001) analysed the thermophilic (60°C) microbial community in a sludge compost made from aerobic activated sludge by forced aeration, they found that it was significantly more varied in batch than chemostat studies, where *Thermoanaerobacterium thermosaccharolyticum* was the dominant detected organism. This showed that the bacterial community in the inoculum can be modified by environmental conditions and is probably less dominated by clostridia in the thermophilic temperature range.

Since clostridia are spore-formers, it is an obvious choice to pre-treat the inoculum in a way that kills all vegetative cells but does not affect spores. Spores of pure clostridial strains used for industrial solvent production were routinely activated by heat-shock. A possible negative side effect of spore selecting pre-treatment is the exclusion of useful facultative anaerobes such as *Enterobacter aerogenes*, which could provide a reduced environment for the obligate anaerobic clostridia.

The most common pre-treatment is heat-shock. Since methanogens and propionate formers do not form spores, they are expected to be de-activated by heat-shock. Comparison of data from two publications by Cohen (Cohen *et al.* 1984; Cohen *et al.* 1985) shows that no propionate production was observed in a continuous acidogenic culture on glucose at pH 6.0, 30°C and various retention times, when the inoculum was pasteurised and the medium sterilised. However, with non-pasteurised inoculum in the same operating conditions propionate production was observed in 9 out of 10 experiments.

It has been observed that heat shock not only kills off all vegetative cells but also aids activation of the spores (section 1.3.1.1). Reported methods of heat-shock are boiling of the inoculum for 15 minutes (sludge, Lay 2000) or baking at 104°C for 2 hours (soil, Van Ginkel *et al.* 2001).

Lay (2000) reports relatively high yields of 2.15 mol hydrogen per mol hexose from continuous experiments on starch, when anaerobic digester sludge, boiled for 15 minutes before the experiment, was used as inoculum. However, from data available to

date, it can not be clearly concluded if heat-treatment improves hydrogen production in practice.

## *1.6 Environmental conditions during cultivation*

Optimisation of environmental conditions for pure cultures would focus on providing optimum conditions for hydrogen production by the chosen strain. In a mixed microbial community this is also an objective but, most of all, conditions need to be found that inhibit hydrogen consumers and allow hydrogen producers to out-compete non-hydrogen producing organisms utilising the same substrates. To date the following operating parameters have been found to influence fermentative hydrogen production: hydrogen partial pressure, temperature, pH, hydraulic retention time in continuous cultivation and oxygen levels.

A preliminary review of factors affecting continuous hydrogen production was published earlier in the preparation of this thesis (Hawkes *et al.* 2002).

### *1.6.1 Hydrogen partial pressure*

As mentioned in section 1.3.1.2 clostridia are only able to dispose of electrons by proton reduction giving hydrogen production if the energy balance is favourable, i.e. at low hydrogen partial pressure. It may also need to be considered that the hydrogen produced by a cell may build up a gas bubble around the cell, effectively separating the cell from substrate in the reactor liquid and causing starvation.

Therefore reduction of the hydrogen partial pressure should be a key aspect of process optimisation. At hydrogen partial pressure above $10^{-4}$ atm clostridia will switch from acetate production as in Equation 1 to an alternative method of electron disposal for NADH regeneration, which results in accumulation of reduced end products such as propionate, lactate and ethanol (Harper and Pohland 1986, Boone and Mah 1987) and a lowering in hydrogen yield.

There are no direct experimental observations of changes in hydrogen partial pressure causing a shift in metabolism, for the main reason that hydrogen partial pressure in the reactor liquid/dissolved hydrogen has not actually been measured. However, it has been

inferred from some observations that hydrogen partial pressure can cause a shift in metabolism. Lay and Noike (1999) for example concluded from batch studies with a mixed culture on cellulose that accumulation of hydrogen was part of the cause for the observed metabolic shift from acid to solvent production at substrate concentrations over 25g $l^{-1}$. In batch studies on solvent production from glucose by *Clostridium saccharoperbutylacetonicum* Brosseau *et al.* (1986) noticed that butanol and acetone were only produced when the formed biogas was prevented from escaping. They concluded from their studies that butanol and acetone were only produced at high hydrogen partial pressure, but volatile fatty acid (VFA) and hydrogen production was not measured, thus there is no direct evidence that hydrogen production was inhibited.

Findings by Zinder (1994) suggest that homoacetogenesis is also influenced by hydrogen partial pressure. They found a thermophilic microorganism that was able to reverse acetogenesis depending on hydrogen partial pressure. At hydrogen partial pressures greater than 500 Pa it was observed to grow acetogenically on hydrogen and carbon dioxide, whilst at hydrogen partial pressures below 40 Pa it could oxidise acetate to hydrogen and carbon dioxide. Unfortunately the organism was not identified and thus no conclusions can be drawn on whether this could occur in a hydrogen producing mixed culture.

There are no reports that propionate production is influenced by hydrogen partial pressure. Furthermore, in fed batch experiments by Inanc *et al.* (1999) propionic acid production by mixed cultures fed with glucose at pH 7 was not changed significantly by artificial reduction or increase of hydrogen partial pressure, nor by application of overpressurized hydrogen gas.

In a natural environment or a single stage anaerobic digester hydrogenotrophic bacteria such as methanogens consume all available hydrogen and thus keep the hydrogen partial pressure low. In an acidogenic reactor hydrogen levels can be kept low artificially. Stirring and gas sparging, or extraction through membranes have been shown to decrease the hydrogen partial pressure.

The influence of stirring on hydrogen partial pressure and type of metabolism has (to my knowledge) only been investigated for batch studies. Unstirred batch cultures of

*Clostridium thermocellum* (37°C, pH 5) for example were found to contain three times more dissolved hydrogen (this was calculated, not measured) than stirred cultures, with butanol productivity being inversely proportional to agitation (Doremus *et al.* 1985). Optimisation of rate of stirring within 250 to 750 rpm improved the hydrogen production rate by over 100% in batch studies with *Citrobacter intermedius* on glucose (without pH control at 34°C) (Brosseau and Zajic 1982a). In batch studies with *Clostridium thermocellum* by Lamed *et al.* (1988) stirring doubled or tripled (depending on bacterial strain) hydrogen yields and acetate concentrations compared to non-stirred conditions, since stirring was associated with a metabolic shift from predominant solvent to acid production. Artificially increased hydrogen partial pressure of $2.5 \times 10^5$ Pa offset the effect of stirring on the ethanol/acetate ratio, which indicates that stirring increased the hydrogen production by decreasing the hydrogen partial pressure in the medium.

Also, Lay (2000) mentions that in continuous experiments with a heat-shocked mixed culture an increase in the agitation speed of a magnetic mixer from 100 to 700 rpm was associated with an increase in hydrogen production from 700 to 1600 l $m^{-3}$ $d^{-1}$. However, since this was not subject of investigation, no details are given, for example if this was associated with a change in metabolism.

Overall, stirring clearly aids hydrogen production. The optimum rate of stirring however can not be directly inferred from the literature, since the effected agitation depends strongly on reactor design.

Although continuous sparging of the reactor during hydrogen production is a relatively simple way of reducing the hydrogen partial pressure, it has not commonly been applied in studies to date. Flushing of the headspace or sparging of the reactor with an inert gas such as argon (Chen *et al.* 2002) or nitrogen (Lay *et al.* 1999) during inoculation has been reported, but with the aim to remove oxygen rather than hydrogen. The effect of continuous sparging with an inert gas has been shown to increase hydrogen yields, but has not been widely investigated. Working in this laboratory Mizuno *et al.* (2000a) showed that sparging of the reactor with nitrogen gas at a flow rate of around 15 times the hydrogen production rate increased hydrogen yields in chemostat experiments with mixed cultures on glucose (35°C, pH 6 and 8.5h HRT) from 0.85 to 1.43 mol hydrogen

per mol glucose, but did not affect the fermentation product distribution. Van Andel *et al.* (1985) on the other hand showed that the butyrate/acetate ratio was significantly decreased (from 16:1 to 2.7:1) due to increasing acetate production, when a pure culture of *Clostridium butyricum* on 10 g $l^{-1}$ glucose (pH 6.0, 30°C, 45 h HRT) was sparged with $N_2$ gas (sparging rate not given). With decreasing HRT, sparging affected the butyrate/acetate ratio considerably less. The effect of sparging on the hydrogen yield was not investigated and can not be inferred from the given data, since the increase in acetate production could be connected to either an increase or a decrease in hydrogen yield (sections 1.3.1.2 and 1.3.3).

To conclude, sparging has not commonly been applied to improve hydrogen yields, but the 68% increase in hydrogen yield reported by Mizuno *et al.* (2000a) is a promising improvement. One problem with sparging to date is that use of a pure industrial gas, such as the nitrogen gas used by Mizuno *et al.* (2000a), increases the process costs and thus may not improve the overall energy balance. For scale up however, this could for example be overcome through the recycling of the remaining process gas for sparging after hydrogen has been stripped. This could be either carbon dioxide from the hydrogen producing reactor or a mix of carbon dioxide and methane, if the effluent of the hydrogen reactor is integrated into a system as suggested in Figure 1-2. A range of membranes for separation of gas components are commercially available and are commonly used to purify hydrogen for use in fuel cells. Teplyakov *et al.* (2002) have used a system of two polyvinyltrimethylsilane membranes to separate components of gas produced in a biohydrogen reactor, achieving gas purities of 90% for hydrogen and 99% for carbon dioxide. Research is currently ongoing to develop low-cost membranes.

### *1.6.2 pH*

pH is probably the most intensively studied parameter in fermentative hydrogen production. Experiments have shown that it has a stronger influence on the dominant metabolism than for example HRT (Zoetemeyer *et al.* 1982c; Horiuchi *et al.* 1999, Lay 2000). Fang and Liu (2002) showed through denaturing gradient gel analysis of 16S rDNA sequences that the bacterial community in a hydrogen producing continuously stirred tank reactor (CSTR) changed significantly with pH in the range of pH 4 to 7.

Although the operating pH should be optimised with the aim of maximum hydrogen production, the amount of alkali required to maintain that pH against the natural acidification caused by VFA production in the reactor also needs to be considered. Zoetemeyer *et al.* (1982c) for example state that the NaOH requirements for an acidogenic CSTR fed on glucose increased by 50% when the operating pH was increased from 4.5 to 5.0 and by more than 100% when the pH was increased from 4.5 to 5.7.

Experiments of hydrogen production from pure clostridial strains (Van Andel *et al.* 1985; Brosseau and Zajic 1982b; Taguchi *et al.* 1995) and enteric strains (Tanisho and Ishiwata 1994; Kumar and Das 2000; Brosseau and Zajic 1982b) are commonly conducted at pH 6.0. Several studies with pure cultures (Heyndrickx *et al.* 1990; Yokoi *et al.* 2001; Kumar and Das 2000) show that throughout the pH range 5.0 to 7.0 hydrogen yields over 2 mol per mol hexose converted are possible. The optimum pH within this range may not only depend on strain, but also on other variables, for example on substrate type for *Clostridium sp. No. 2* (Taguchi *et al.* 1994), or on nutrient limitation and HRT for *Clostridium butyricum* (Heyndrickx *et al.* 1990).

For mixed mesophilic microflora most investigations of optimum pH for hydrogen production point towards the range of pH 5 to 6 (Table 1-2), independent of HRT and substrate type.

***Table 1-2. Optimum operating pH for mesophilic cultures reported in the literature***

| pH range tested | Opt. pH | Inoculum | Substrate | HRT [h] | Temp. [°C] | Max. yield [mol $H_2$ $mol^{-1}$ hexose] | Reference |
|---|---|---|---|---|---|---|---|
| 4.0-6.0 | 5.2 | Heat treated anaerobic sludge | Starch | 17 | 37 | 2.15 | (Lay 2000) |
| 4.5-7.5 | 5.5 | Heat treated potato soil | Sucrose | Batch | 37 | 2.4 | (Van Ginkel *et al.* 2001) |
| 4.0-7.0 | 5.5 | $H_2$ producing sludge | Glucose | 6 | 36 | 2.1 | (Fang and Liu 2002) |

Metabolic changes and population changes have been observed when the pH was increased to near neutral or decreased to below pH 5.

Methanogenesis can be excluded either by pH less than 6 (see section 1.3.5.) or short retention times. Fang and Liu (2002) observed development of methane production in a mesophilic hydrogen producing mixed culture when the pH was increased to 6.0, even at the short HRT of 6h. This suggests that reduction of HRT alone might not be sufficient to inhibit methanogens at near-neutral pH, as methanogens may be able to attach to reactor parts and thus increase their own retention time. Zoetemeyer *et al.* (1982c) report strong lactic acid production at pH above 6 and increasing acetate/formate/ethanol production with increasing pH at and above pH 7 from mesophilic CSTR experiments with a mixed culture on glucose. Hydrogen production was not measured, but the total gas production decreased steadily with increasing pH in the range of pH 6 to 8. Horiuchi *et al.* (1999) and Horiuchi *et al.* (2002) report that the dominant metabolism in a mesophilic chemostat culture (anaerobic digester sludge on glucose) changed from butyrate/acetate to acetate/propionate production when the pH was increased to 8 or above. From the relatively long time span that this shift in metabolism took (120 to 150 hours at 10 h HRT) the authors conclude that a shift in microbial population must have occurred. Onset of propionate production (16% of products on carbon basis) was also observed by Fang and Liu (2002), when the pH was increased to 7.0.

From these publications no general pattern for metabolic or population shift with increasing pH emerges, which indicates that other parameters such as the inoculum or the HRT might be determining what the butyrate-acetate producing culture changes to. However, it can be concluded that the butyrate-acetate metabolism is less competitive at near neutral pH than at pH 5 to 6.

At pH less than 5 it was observed by Lay (2000) that hydrogen production switched to solvent production in a mixed culture on soluble starch. As discussed in section 1.3.1.3 pH 4.3 to 5.5 is commonly given as trigger value for solvent production in batches. Fang and Liu (2002) also report from a hydrogen producing mixed chemostat culture on glucose (6h HRT, 36°C) that the glucose conversion decreased at pH below 5.5.

Ren *et al.* (1997) on the other hand report from CSTR experiments with a mixed culture and molasses at 30°C, that gas consisting of 32.5 to 49.5% hydrogen was produced at

pH range 4.3 to 4.6, whilst no gas was produced at pH 4.9. The hydrogen yield can not be accurately calculated from the data given, but can be estimated as in the range of 0.85 to 1.4 mol hydrogen per mol hexose added from an earlier publication (Ren *et al.* 1995) describing the same experiments. Their reason for setting the operating pH so low was to avoid propionic acid fermentation, which they claim is dominant in the pH range 5 to 6. There is no clear proof for their theory that propionate production out-competes hydrogen production at pH 5 to 6 in the data they provide. However, they report stable continuous hydrogen production in association with ethanol and acetate production at pH around 4.5. Results by Hwang *et al.* (2004) support the theory by Ren *et al.* (1997) in that strong propionate production (up to 17.6 mmol $l^{-1}$) developed at pH 6.0 in semi-batch experiments with a mixed culture on 5 g $l^{-1}$ glucose, whilst at pH 4.0 to 5.0 propionate concentrations were below 3.4 mmol $l^{-1}$ and ethanol and acetate production was dominant. However, stable hydrogen production was not observed at any pH in this experiment.

From the information available in the literature it can therefore be concluded that pH 5 to 6 probably allows maximum hydrogen production by a range of species. However, if product inhibition does not occur, pH below 5 might also be suitable for hydrogen production and reduce alkali requirements.

### *1.6.3 Temperature*

Fermentative hydrogen production has been reported from mesophilic and thermophilic operation. There is considerably more information available on mesophilic continuous hydrogen production than on thermophilic operation, and there are also more mesophilic clostridial species known (Ljungdahl *et al.* 1989).

In the mesophilic range most experiments with successful hydrogen production from mixed cultures were controlled at 35°C (Lin and Chang 1999; Mizuno *et al.* 2000a), 36°C (Fang and Liu 2002) and 37°C (Lay 2000, Sung *et al.* 2002). From pure cultures of *Clostridium butyricum* (Yokoi *et al.* 2001) and *Clostridium pasteurianum* (Brosseau and Zajic 1982b) yields of 2.4 mol hydrogen per mol hexose have been reported at 37°C in batches. However, high hydrogen yields of 1.8 and 2.3 mol per mol hexose have also been reported at 30°C, in continuous operation from pure cultures of *Clostridium*

*butyricum* on glucose by Van Andel *et al.* (1985) and Kataoka *et al.* (1997) respectively. None of these studies however focused on temperature optimisation. Taguchi *et al.* (1994) investigated hydrogen production at 31, 36 and 41°C by *Clostridium* sp. No. 2 from glucose, arabinose and xylose in batches. The experiments showed slight variation in hydrogen yields with temperature in this range. Hydrogen yields were lowest at 31°C for all three substrates (2.0 to 2.5 mol per mol hexose/pentose) and could be increased by 0.1 to 0.4 mol per mol substrate at the higher temperatures of 36 and 41°C. Negligible increase in yields (up to 0.1 mol per mol hexose/pentose) were observed when raising the temperature from 36 to 41°C.

For pure cultures of enteric bacteria optimum temperatures for hydrogen production have been investigated in batch studies. Oh *et al.* (2003) report that 36°C allowed maximum hydrogen production activity in the range 25 to 40°C from *Citrobacter* sp. Y19, Yokoi *et al.* (1995) report 38°C as giving maximum hydrogen production in the range 29 to 43°C for *Enterobacter aerogenes* strain HO-39, and Kumar and Das (2000) report that maximum hydrogen yields were obtained at 36°C in the range 15 to 45°C for *Enterobacter cloacae* IIT-BT 08.

From these results can be concluded that in the mesophilic range good hydrogen yields can be achieved at around 35 to 38°C, but little work to date has focused on temperature optimisation. There are some indications that lower temperatures may also achieve good yields: For example the studies by Van Andel *et al.* (1985) and Kataoka *et al.* (1997) at 30°C mentioned above, and studies by Fang, Liu *et al.* (2002) with a granular sludge and sucrose at 26°C, achieving hydrogen yields of 2.3 mol per mol hexose.

There are also indications that the optimum operating temperature for hydrogen production by a mixed culture may vary, depending on other operating parameters. Zoetemeyer *et al.* (1982a) investigated effects of various temperatures on acidogenic conversion of glucose (activated sludge inoculum, pH 5.8). In their experiments at 10 h HRT butyrate production was significantly weaker and propionate production stronger at 37°C than at higher or lower temperatures, whilst at 2 h HRT this was not observed. This suggests that the optimum operating temperature for a mixed culture may depend on the HRT.

In the thermophilic range Ueno *et al.* (1996) report successful continuous hydrogen production at 60°C with a mixed culture on sucrose, achieving maximum yields of 2.5 mol hydrogen per mol hexose at pH 6.8 and 12 h HRT. Zoetemeyer *et al.* (1982a) report optimum glucose conversion rates at 52°C for an acidogenic reactor (pH 5.8) with activated sludge on glucose, achieving 80% glucose conversion at 1.4 h HRT. However, this was associated with ethanol as the main product, whilst butyrate was the main product at temperatures below 50°C, suggesting that the optimum temperature for glucose conversion may be different to that for hydrogen production.

Experiments to date do not allow clear conclusions as to whether mesophilic or thermophilic operation is more suitable for hydrogen production. Experiments by Zoetemeyer *et al.* (1982a) with an acidogenic culture (not focused on hydrogen production) led to the conclusion that mesophilic operation of the reactor was more stable, but thermophilic glucose conversion rates were higher: retention times could be shortened by 40% without washout at thermophilic compared to mesophilic operation.

The obvious advantage of mesophilic operation is that the energy required to heat reactor and feed is significantly less. The feed heat requirements for a reactor are represented by Equation 18 (Srivastava 1987), which shows that the energy requirement is directly proportional to the required increase from storage to operating temperature.

$$Q_i = WC_p (T_d - T_a) \qquad \textit{Equation 18}$$

With $Q_i$ = feed heat requirement

W= total weight of the feed

$C_p$ = specific heat of the feed

$T_d$ = digester operating temperature

$T_a$ = ambient temperature.

Thermophilic operation is therefore particularly of interest for waste/co-products of industrial processes, where these leave that process at higher temperature, for example brewing waste, or where there is waste heat. Alternatively, some of the heating energy can possibly be recovered from the reactor effluent via heat exchangers. Some recent publications on hydrogen production from food waste suggest that for batch and semi-batch operation on complex substrates thermophilic operation may be preferable.

Valdez-Vazquez *et al.* (in press) report from semi-batch operated experiments with anaerobic digester sludge on kitchen and paper waste (freeze dried and milled) that a maximum of 3.2 mol hydrogen per mol hexose was produced at 55°C compared to 1.5 mol hydrogen per mol hexose at 35°C. Similarly, Shin *et al.* (2004) report from batch experiments with a mixed acidogenic culture on food waste from a dining hall, that 1.8 mol hydrogen per mol hexose were produced at 55°C, whilst hydrogen production was negligible at 35°C.

### *1.6.4 Hydraulic retention time*

Generally the hydraulic retention time (HRT) is kept as short as the growth rate of the hydrogen producing culture allows, to exclude more slowly growing organisms such as methanogens and possibly some homoacetogens (Ueno *et al.* 1996). The minimum retention time required depends strongly on the complexity of the substrate.

On glucose, the simplest carbohydrate substrate, retention times as short as 3.5 hours are reported to allow >90% glucose conversion with dominant butyric acid production in a chemostat reactor inoculated with activated sludge (10 g $l^{-1}$ glucose, pH 5.7 and 30°C; Zoetemeyer *et al.* 1982c). Lin and Chang (1999) however report from experiments with mixed cultures and 20 g $l^{-1}$ glucose that only 80% of glucose was consumed at 6h HRT (pH 5.7 or 6.4 and 35°C), which may indicate that for higher feed concentrations longer retention times are required. Successful hydrogen production by mixed cultures on glucose has been reported at 6 h HRT (at pH less than 6) (Fang and Liu 2002) and 8.5 h HRT (Mizuno *et al.* 2000a). Fang and Liu (2002) also report growth of methanogens at 6 h HRT in a mixed culture on 7 g/l glucose at pH 6 or above. However, a problem with methanogenesis at such short retention times has not been reported elsewhere, and it might therefore be possible that in these experiments methanogens were able to grow on reactor walls or parts, thus avoiding washout.

For sucrose 6h HRT was found to be the lower limit by Chen and Lin (2001). At shorter retention time washout of the mixed hydrogen producing culture (pH 6.7) was observed. Optimum retention times of 8.0 h (Chen and Lin 2003), 8.7 h (Chen *et al.* 2001), 12 h (Ueno *et al.* 1996) and 13.7 h (Liu and Fang 2002) are reported for maximum hydrogen

yields from sucrose and inhibition of methanogenesis. Chen and Lin (2001) for example report washout of methanogens at 13.3 h HRT in a mixed culture on sucrose at pH 6.7.

For more complex substrates such as cellulose and starch enzymatic hydrolysis is the rate limiting factor. Giallo *et al.* (1985) for example report from batch studies with *Clostridium cellulolyticum* that their doubling time on cellulose is 24 h in comparison with 10 h on glucose. Statistical analysis of 23 experiments by Lay (2000) on continuous hydrogen production from soluble starch by a mixed culture indicates an optimum HRT between 12.7 and 20.2 hours, with 17 h HRT giving maximum hydrogen yields of 2.15 mol hydrogen per mol hexose. Ueno *et al.* (2001) report mesophilic continuous hydrogen production from cellulose by a mixed culture at the long HRT of 3 days (pH 6.4), without encountering problems with methanogenesis. Since continuous hydrogen production from complex substrates is to my knowledge not reported elsewhere, more research into optimum retention times on these substrates is required.

### *1.6.5 Redox potential*

Clostridia are obligate anaerobes, since they have very little or no ability to produce superoxide dismutase (Gregory *et al.* 1978) or catalase (Buchanan and Gibbons 1975) and thus very little or no ability to deactivate toxic products of oxygen such as the hydroxyl and superoxide radicals (White 2000). In conventional anaerobic digestion the microbial community includes some facultative anaerobes such as *Enterobacter aerogenes*, which consume any oxygen that might be present in the reactor and thus keep the redox potential low. However, in studies with pure cultures of clostridial strains or where a mixed culture was heat-shocked and thus consists only of spore formers, chiefly clostridia, hydrogen production may be inhibited by high redox potential. Yokoi *et al.* (1998b) for example show that aeration of a pure culture of *C. butyricum* for 20 minutes stopped hydrogen production immediately, and the culture did not recover at all. The authors concluded that in this case the clostridial cells were irreparably damaged. When L-cysteine, a reducing agent, was added continuously throughout a repeat experiment, hydrogen production re-started after 15 hours. If *Clostridium butyricum* was co-cultured with the facultative anaerobe *E. aerogenes* instead of continuous L-cysteine addition, hydrogen production showed a short

interruption, but recovered within 30 minutes, showing that the co-culture was little affected by air.

Typical redox potentials associated with butyrate/acetate metabolism and fermentative hydrogen production are not easily obtained from the literature, as the method for measuring redox potential is rarely stated. Most commercially available redox probes measure a relative redox potential against a Ag/AgCl reference system. To convert this redox potential to the standard redox potential which would be obtained against the standard hydrogen reference electrode, at 25°C a voltage of +200 mV would have to be added to the Ag/AgCl electrode readings. This value is temperature dependent and decreases by 1.2 mV per 1°C temperature increase (APHA 1995). Therefore redox potentials given in the literature can not be directly compared unless the method of measurement and any adjustments to the measured values are stated. Bearing this in mind, butyrate-acetate metabolism and hydrogen production are reported to be associated with redox potentials of –500 to –550 mV for a pure culture of *Clostridium butyricum* (Kataoka *et al.* 1997, method not given), -300 mV for a mixed culture (Cohen *et al.* 1984, reported as measured with Ag/AgCl electrode) and a pure culture of *Clostridium pasteurianum* (Brosseau and Zajic 1982b, method not given), –250 mV for *Clostridium acetobutylicum* (Kim *et al.* 1988, Ag/AgCl electrode), –241 to –256 mV for the "ethanol type fermentation" described by Ren *et al.* (1995, method not given) and -100 mV for a pure culture of *Citrobacter intermedius* (Brosseau and Zajic 1982b). Hydrogen production appears therefore to be associated with generally negative redox potentials.

The experiments with mixed cultures described by Cohen *et al.* (1985) suggest that the redox potential may vary with species, as it increased from -300mV to –170mV when the dominant species in the culture changed from clostridia to *Selenomonas* sp. A change in redox potential may also be associated with a change in dominant culture metabolism. For *Clostridium acetobutylicum* for example maximum solvent production was found to be associated with a slightly higher redox potential of -230 mV compared to –250 mV for maximum butyrate production (Kim *et al.* 1988). Further, Lin and Jo (2003) report from sequencing batch experiments with waste activated sludge on sucrose, that the redox potential decreased with increasing loading rate, from –270 mV at 40 kg COD $m^{-3}$ $d^{-1}$ to –590 mV at 120 kg COD $m^{-3}$ $d^{-1}$. The redox potential therefore

appears to be influenced by several parameters. Results by Yokoi *et al.* (1998b) described above suggest that optimisation of the redox potential may be essential for stable hydrogen production. However, this has not been further investigated to date. From Cohen *et al.* (1985) for example it cannot be determined whether the change in redox potential was a cause for rather than an effect of the change in culture population. If a change in redox potential could be demonstrated to cause a change in population/metabolism, redox potential could be a useful control parameter.

## *1.7 Substrate*

The ideal substrate should allow high hydrogen yields, have minimum production costs and require minimum pre-treatment.

### *1.7.1 Type of organic matter: carbohydrate, protein or fat?*

Whilst there are numerous reports of hydrogen production from carbohydrates such as glucose, sucrose and starch, continuous hydrogen production from fat or protein has not been reported to date for mesophilic or thermophilic cultures. Clostridia, which are thought to be the dominant species in mesophilic hydrogen production, can metabolise a wide range of substrates, including polysaccharides such as starch, cellulose, hemicellulose and pectins, as well as proteins and amino acids (Schlegel 1993). However, the acetate-butyrate metabolism, the concept on which this project is based, is essentially a carbohydrate-fermenting metabolism.

The suitability of individual organic fractions of municipal solid waste for hydrogen production has for example been investigated by Okamoto *et al.* (2000) and Lay *et al.* (2003), both using a mixed microflora (anaerobic digester sludge, heat-treated for 15 min) at 37°C and initial pH 7. Okamoto *et al.* (2000) give yields in the form of "hydrogen production potentials" as shown in Table 1-3. These "potentials" agree with findings by Lay *et al.* (2003) that 15 to 30 times more hydrogen was produced from rice and potato (carbohydrate) than from egg, meat or chicken skin (protein and fat) in batch studies with 100 g TS substrate per litre.

*Table 1-3. Hydrogen production potentials of different substrates (Okamoto et al. 2000)*

| Substrate | Treatment | Potential (ml/g-VS) |
|---|---|---|
| Cabbage | Blended | 26.3-61.7 |
| Carrot | Blended | 44.8-70.7 |
| Rice | Boiled and blended | 19.3-96.0 |
| Egg | Boiled and blended | 2.6-7.1 |
| Lean meat | Boiled and blended | 2.5-7.7 |
| Chicken skin | Blended | 3.6-10.2 |
| Fat | Blended | 4.4-11.1 |

Both studies show that carbohydrates are significantly more suitable for hydrogen production by anaerobic spore formers than lipids and proteins. For example, 4% of carrot, but only 0.2% fat and 0.1% lean meat were converted to hydrogen gas (based on COD). Also, the lag phase for hydrogen production from fat was more than five times longer than that from carbohydrates or protein (Okamoto *et al.* 2000). Experiments by Mizuno *et al.* (2000b), investigating the ability of a mixed mesophilic culture to produce hydrogen from bean curd wastewater in batches (pH 6.0 and 35°C), also showed that the culture degraded carbohydrate preferentially to protein. The substrate in these studies contained 5.01 g $l^{-1}$ protein and 3.75 g $l^{-1}$ carbohydrate. Hydrogen yields of 2.54 mol $H_2$ per mol hexose were obtained from the carbohydrate, but almost none of the protein, of which 65% was soluble, was degraded.

Similar observations were also made in a thermophilic mixed culture by Yu and Fang (2002). They report from batch studies with anaerobic digester sludge on powdered milk at pH 5.5 that the carbohydrate in the substrate was converted first, and this was associated with hydrogen and butyrate production. Over 70% of the protein was also converted, but only after the carbohydrate was depleted, and the protein consumption was not associated with hydrogen production. Of the lipid in the substrate only 12 to 30% was converted.

Since there is no shortage of carbohydrate rich crops or wastewater and it has been shown that continuous hydrogen production is possible from carbohydrates, only carbohydrate rich substrates have been considered in this thesis and will be reviewed.

### *1.7.2 Carbohydrates and crops for biohydrogen production*

Most studies on fermentative hydrogen production have focused on mono- and disaccharides, since these are more readily degradable and allow for shorter retention times than polysaccharides.

Whilst soluble sugars have been reported to be the preferred substrate, and inhibition of cellulose and xylose degradation has been observed when mono- or disaccharides were added (Mitchell 1998), some clostridial strains, such as *Clostridium polysaccharolyticum*, which only converts polymers of cellulose and starch (Ljungdahl *et al.* 1989), specialise in degradation of complex substrates. It can therefore be assumed that a mixed culture, such as present in anaerobic digester sludge, should be able to convert a mixture of mono-, di- and polysaccharides to hydrogen, even though the culture may show overall diauxic growth, converting simple sugars first.

To make the process of fermentative hydrogen production economically feasible, substrate costs have to be kept to a minimum. In the solvent industry for example, molasses, the main substrate, made up about 63% of the total cost of solvent production (Duerre 1998; Biebl 1999). Therefore research into fermentative hydrogen production should focus in the long term on low cost agricultural and waste products which are predominantly polysaccharides. The use of cheaper carbohydrate sources such as low-grade potatoes and potato wastes, maize or rye (Duerre 1998), apple pomace and Jerusalem artichokes (Biebl 1999) was also suggested for the solvent industry, but where complex substrates were used for solvent production, they were pre-treated. Potatoes for example were pre-treated with steam-explosion and α-amylase (Duerre 1998) and Jerusalem artichokes with enzymatic hydrolysation (Biebl 1999). Although pre-treatment may have to be considered for fermentative hydrogen production to allow use of a wider range of substrates, it should be kept to a minimum to keep costs low. Studies on thermophilic hydrogen production from pure ball milled cellulose (Ueno *et al.* 2001) and on mesophilic hydrogen production from pure soluble starch (Lay 2000) have shown that continuous hydrogen production from polysaccharides is possible in principle, but process optimisation is required.

When choosing energy crops several aspects have to be considered (Venturi and Venturi 2003):

- Suitability for fermentation (carbohydrate content and composition, required pre-treatments)
- Suitability for the considered environment (soil, climate, topography)
- Ease of introduction (suitability for existing rotation, knowledge and technology for growing and harvesting)
- Profitability (energy balance, income for grower)

The three substrates used for experiments described in this thesis were chosen with these aspects in mind and will be introduced in the following sections.

### *1.7.3 Starch*

The main sources for commercial starch extraction are potato tubers, maize grains and wheat flour (Kennedy and White 1983). For this project a co-product of the wheat starch industry was used as substrate. In an evaluation of wheat as a crop for ethanol production by Venturi and Venturi (2003) wheat is shown to have several advantages: it is already widely grown with well established technology and low transport costs. It is also easily introduced to new rotations and expected future yield gains are high. The UK currently produces around 15 million tonnes of wheat on ~2 million ha (Venturi and Venturi 2003) each year, of which 31% is used to make flour (HGCA 2005). 5.6 million tonnes wheat were processed to 4.5 million tonnes flour with 1.1 million tonnes co-products in the UK in 2000. These co-products are chiefly wheatfeed (bran) with a small and variable quantity of low grade flour, which consists mostly of starch. Low grade flour does not give the required performance when used in baking. It arises in variable amounts and may be blended into less sensitive flours, used in dog biscuits, or landfilled.

Starch has two major components: amylose and amylopectin, both of which are polymers of D-glucose. Amylose is a predominantly linear polymer of molar weights in the range of $10^5$ - $10^6$. Amylopectin is a highly branched polymer with unit chain lengths of 17-26 glucose residues and molar weights in the range of $10^7$-$10^9$. Linear linkages between glucose units are $\alpha 1 \rightarrow 4$ linkages, branch points also have $\alpha 1 \rightarrow 6$ linkages (Galliard 1987). Amylose forms a random coil in neutral conditions, but in presence of complexing agents it forms a regular helical structure.

Starch is produced in large quantities by plants, in some of which it is the main food-reserve polysaccharide (Kennedy and White 1983). In the various parts of plants starch is found in concentrations ranging from a few percent to 90 percent dry weight. Kim *et al.* (2003) report that non-genetically modified wheat starch has a typical amylose content of 26%. Non-carbohydrate components present in starch are inorganic substances, lipids, proteins and phosphate groups. These components can be residues on the surface of the starch granule, but can also be internal components (particularly lipids), forming complexes with amylose (Galliard 1987). The UK Flour Advisory Bureau (FAB 2005) gives the average composition of wholemeal wheat flour as 63.9% carbohydrate (amylose plus amylopectin), 12.7 % protein, 2.2% fat and 9% fibre. Rank Hovis gives the average composition of low grade wheat flour as 70.1-73.4 % carbohydrates, 15% protein, 1.6-3.2% fat and 13% water (White 2004).

Experience in hydrogen production through fermentation of starch is so far very limited. However, published data exists which shows that hydrogen production from starch is possible. Lay (2000) for example used pure soluble starch as substrate in 23 short (approximately 4 day) continuous experiments with a heat treated mixed culture at 37°C, investigating the effect of feed concentration in the range of 2.5 to 7.5 g $l^{-1}$ (with corresponding changes in HRT from 10 to 30 h to keep the organic loading rate constant at 6.0 kg starch $m^{-3}$ $d^{-1}$) and pH in the range of 4.0 to 6.5. Maximum yields of 2.14 mol hydrogen per mol hexose were reported at pH 5.2 and 17 h HRT. In repeat batch studies conducted by Yokoi *et al.* (2002) a culture of *Clostridium butyricum* and *Enterobacter aerogenes* produced 2.7 mol hydrogen per mol hexose from a starch residue. The starch residue used was obtained after extraction from sweet potatoes.

Taguchi *et al.* (1997) state in their patent that with their method using *Clostridium beijerinckii* 7.7 mol hydrogen per mol hexose could be produced from starch (no details given on starch properties) in batches. These yields however exceed the theoretical maximum of 4 mol hydrogen per mol hexose obtainable through the acetate/butyrate metabolism, and must therefore either be unrealistic or involve other, unknown, hydrogen production mechanisms.

### *1.7.4 Sugarbeet and sucrose*

Sugarbeet, a temperate climate biennial root crop, has been included in a list of energy crops by the UK Department for Environment, Food and Rural Affairs (DEFRA 2005). It is of interest as a substrate for hydrogen production, as it is already commonly present in European crop rotation and well established as a substrate for fermentation, e.g. to bioethanol, due to its high water and sucrose content. Sucrose ($C_{12}H_{22}O_{11}$) is a crystalline disaccharide of fructose and glucose. Sugarbeet grown in the UK currently yields around 54 t wet weight $ha^{-1}$ (IENICA 2005) with a water extractable sucrose content of 170g per kg wet beet (British Sugar 2005). Typical dry matter composition of sugarbeet is shown in Table 1-4. Purdue University (2004) gives a dry matter content of English beet of 23.4%.

***Table 1-4. Composition of the whole sugarbeet root***

| Components [% of total dry matter] | | | | | Reference |
|---|---|---|---|---|---|
| Crude ash | Crude protein | Ether extract | Crude fibre | Sucrose | |
| 5.0-8.1 | 4.7-6.8 | 0.3-0.8 | 4.9-6.3 | 64.7-70.0 | OECD (2002) |
| 8.1 | 6.8 | 0.6 | 5.4 | 64.7 | Weiland (2003) |
| 6.0-8.7 | 12.6-14.3 | 0.8-1.6 | 6.3-9.0 | 77.9-79.4 | Purdue University (2004) |
| | 7.2 | 0.3 | 5.6 | 73.6 | Aulrich *et al.* (2002) |

It appears that the nutrient concentration in sugarbeet is highly variable (Table 1-5), presumably depending on age of the crop and growth conditions.

***Table 1-5. Nutrients in sugarbeet***

| Nutrients [% w/w of total dry matter] | | | | | | Reference |
|---|---|---|---|---|---|---|
| Na | K | P | Ca | Fe | α-amino-N | |
| 0.06 | 0.2 | | | | 0.04 | Eckhoff and Bergman (2001) |
| 0.09 | 0.6 | 0.07 | 0.06 | 0.09 | | Purdue University (2004) |
| 0.007 | 0.13 | | | | 0.008 | Roggo *et al.* (2003) |
| 0.1-0.2 | 1.3-1.7 | 0.3-0.5 | | | 0.2-0.3 | OECD (2002) |

Currently sugarbeet growth in the UK is restricted to areas within delivery distance of a sugar refinery. Only 3 % of the arable land, 135,000 ha (British Sugar 2005) to 188,000 ha (average for 1996 to 2000; Venturi and Venturi 2003), is allocated to sugar beet in the UK. If used as an energy crop with on-site fermentation facilities, sugarbeet could be more widely grown.

In temperate climates sugarbeet is the main crop from which sucrose is extracted commercially. For industrial extraction of the sucrose juice (extractable to >90%), the sugarbeet is washed and sliced into cossettes, which are extracted with water at ~ 70 °C for 100 minutes (Eckhoff and Bergman 2001). The sucrose juice is purified with lime and carbon dioxide, thickened through evaporation and then crystallised through further evaporation. The side-product lime can be used as fertiliser. Typically, 120 to 200 g sucrose per kg wet beet are extracted (Mahn *et al.* 2002; IENICA 2005; OECD 2002). Table 1-6, giving an example of composition of a sugarbeet extract, shows that the dry solids in this extract consist 95% of sucrose.

***Table 1-6. Composition of raw sugar beet extract (Ogbonna et al. 2001)***

| Component | Concentration [% w/w] |
|---|---|
| dry solid | 17.3 |
| sucrose | 16.5 |
| raffinose | 0.07 |
| monosaccharides | 0.15 |
| polysaccharides | 0.019 |
| Total N | 0.105 |
| Na | 0.015 |
| K | 0.125 |
| $PO_4$ | 0.047 |

After sucrose extraction, a residue of 45 to 70 g pulp per kg wet beet (IENICA (2005) and Hutnan *et al.* (2000) respectively) remains, which consists mainly of insoluble carbohydrates. Coughlan *et al.* (1985) for example report a typical pulp composition of 22-30% cellulose, 24-32% hemicellulose (essentially arabinan) and 24-32 % pectin. The pulp is commonly pressed and dried to 85-90% dry matter to be used as feed for ruminants and pigs. Molasses are a further by-product of the industrial sugar refining process, of which 50% is sugar and 20% non-sucrose organic matter, particularly non-protein nitrogen containing substances (e.g. betaine), accompanied by amino acids and conversion products of glutamine (OECD 2002).

Although clostridia are natural contaminants in sugarbeet extraction towers, and their hydrogen production is even thought to be the cause of an explosion in a German sugar factory (Meisel *et al.* 1997), fermentative hydrogen production directly from sugarbeet has not been investigated to date. However, hydrogen production has been reported from pure sucrose (Fang and Liu 2001; Chen *et al.* 2001; Liu and Fang 2002; Sung *et al.* 2002), sugar factory wastewater (Ueno *et al.* 1996) and dilute molasses (Tanisho and

Ishiwata 1994), achieving yields in the range of 0.8 (Chen and Lin 2001) to 2.5 (Ueno *et al.* 1996) mol hydrogen per mol hexose. Most experiments with sucrose focused on mesophilic cultures, operating at 26°C (Fang and Liu 2001) to 37°C (Sung *et al.* 2002), but Ueno *et al.* (1996) demonstrated that thermophilic hydrogen production from a sugary wastewater was also possible. For mixed microflora, reactors were operated at pH 5.5 (Fang and Liu 2001; Van Ginkel *et al.* 2001; Sung *et al.* 2002) and pH 6.7, but at pH 6.7 methane production was detected for HRT over 13.3 h (Chen and Lin 2001; Lin and Jo 2003). For pure cultures of *Enterobacter cloacae* (Kumar and Das 2000) and *Enterobacter aerogenes* (Tanisho and Ishiwata 1994) reactors were operated at pH 6.0. From data available to date it can be concluded that hydrogen production from sucrose by a mixed culture should be possible in a range of operating conditions, but conditions are not yet optimised. Since sugarbeet has a high sucrose content, which can be extracted in water, operating conditions allowing hydrogen production from sucrose should also be suitable for hydrogen production from sugarbeet.

### *1.7.5 Grass*

66% (12.3 million ha) of total UK agricultural land is grass. As it is abundant in the UK and the required technology is well established, it would be a desirable substrate for hydrogen production during spring and summer, when sections of grassland could be purpose cut several times a season, and the harvested fresh grass directly used as fermentation substrate.

Commercial seed mixtures for pasture contain predominantly ryegrass species, accompanied by Timothy grass and clover. Ryegrasses are the most widely used grass species in UK agriculture (IGER 2004), giving a yield of 11 to 13 t dry matter per ha (Morris 2004).

In the UK fresh grass has a dry weight of approximately 5 to 20 %. The major part of the dry weight consists of carbohydrates, which can be divided into the structural carbohydrates, forming the plant cell wall and accounting for most of the grass dry weight, and the non-structural or reserve carbohydrates, which are highly variable in their abundance. The cell wall consists of polymer networks, which are linked to each other through various types of bonds and thus form a rather complex structure. The composition of the cell wall varies in different parts of the wall, the different types of

cell, in different plant species and probably also at different stages of the cell cycle (Brett and Waldron 1996). The main components are cellulose, hemicellulose, phenolics & lignin, protein and pectin, the carbohydrate fraction consisting in general of 90% hexose and 10% pentose (Morris 2004). The non-structural carbohydrates are all hexoses. Table 1-7 summarises information on typical grass composition given in the literature.

***Table 1-7. Typical carbohydrate components of grasses***

| Grass | Components [% of grass dry weight] | | | | | | Reference |
|---|---|---|---|---|---|---|---|
| | Non-structural carbohydrates | Structural carbohydrates | | | Structural non-carbohydrate | | |
| | | cellulose | hemi-cellulose | pectin | protein | lignin | |
| *Lolium perenne* | | 25-40 | 15-35 | | 7-20 | 5-12 | Morris (2004) |
| *Lolium perenne* | 13 – 18 | | | | | | Boudon *et al.* (2004) |
| *Dactylis glomerata* (forage grass) | | 29.5 | 23.7 | 0.6 | 10.9 | 2.6 | Yahaya *et al.* (2001) |
| Average of American pasture grasses 2000 to 2003 (over 290 samples) | 9 | 24 | 31.5 | | | 4.9 | Dairy One (2004) |
| American forage grass | | | | 2-5 | | | Hall (2004) |
| Dutch forage grass | 9.8 | 21.1 | 22.5 | 5.5 | 10.9 | 6.2 | Gaillard (1962) |

### 1.7.5.1 Non-structural carbohydrates

Non-structural carbohydrates in forage grasses include glucose, fructose, sucrose, starch and fructan, which consists of up to 200 fructose units linked through β(2→1) and β(2→6) bonds. 90% of the major non-structural carbohydrate in grasses is fructan, less than 3% sucrose and less than 1% glucose and fructose (Morris 2004). The absolute concentrations of these non-structural carbohydrates are highly variable and depend on time of day and year. Cairns (2003) for example reports that the concentration in perennial ryegrass (*Lolium perenne* cv. Aurora) in the UK ranged from less than 2 g per kg wet grass at night to 9 g per kg wet grass at noon. Overall nonstructural carbohydrate

concentrations of 6% dry weight (Dairy One 2004) to 18% dry weight (Boudon *et al.* 2002) have been reported. In the UK ryegrass has also been specifically modified to contain up to 35% fructan (per total dry weight) (Cairns 2003).

Whilst glucose, fructose and sucrose are readily digestible by hydrogen producers (see section 1.7.2), to date no information is available on the ability of hydrogen producers to hydrolyse fructan. It is therefore possible that without pre-treatment of the substrate the majority of non-structural carbohydrates is not readily converted to hydrogen.

#### 1.7.5.2 Cellulose

Cellulose, an unbranched β 1,4-glucan with a chain length of over 15 000 glucose units, forms long thin microfibrils in the cell wall. The cellulose chains are held in a crystalline or paracrystalline lattice within the microfibril. This lattice is stabilised by both intramolecular and intermolecular hydrogen bonds (Brett and Waldron 1996; Mitchell 1998). Degradation of cellulose depends on the porosity of the substrate and the cellulose fibre crystallinity (Sun and Cheng 2002).

Cellulolytic clostridia are known both in the mesophilic and thermophilic range, but not many clostridia can degrade cellulose. Lee *et al.* (1985a) for example report that only two strains (both *Clostridium acetobutylicum*) out of 21 clostridial strains tested had cellulolytic enzymes. Even these two strains (NRRL B527 and ATCC 824) could not break down crystalline cellulose, and the decomposition of microcrystalline cellulose (Avicel) was a very slow process, as hydrolysis of the cellulose was the rate-limiting factor. B527 for example only hydrolysed 4% of 5 g $l^{-1}$ untreated microcrystalline cellulose in 29 h of incubation and 30% of 5 g $l^{-1}$phosphoric acid swollen cellulose in 24 hours. *Clostridium cellulolyticum* H10 took 2 weeks to degrade 70% of insoluble pure cellulose in batch studies with initial cellulose concentrations of 0.6 to 7.6 g $l^{-1}$ (Giallo *et al.* 1985). Hydrogen production by heat-treated anaerobic digester sludge on microcrystalline cellulose (Avicel type Funacel powder) had a lag phase of around 4 days (Lay and Noike 1999).

Thermophilic continuous hydrogen production from cellulose by a mixed microflora has been investigated by Ueno *et al.* (2001). They report hydrogen yields of 2 mol hydrogen per mol hexose from powdered cellulose at 60°C, pH 6.4 and 3 day HRT using forced

aerated sludge compost as inoculum. When 5 g $l^{-1}$ peptone were added to 10 g $l^{-1}$ cellulose, 90% of substrate was converted. However, continuous operation was stopped after 2.7 retention times. Reasons for this were not given. Continuous mesophilic hydrogen production from cellulose has not yet been reported. Lay (2001) report hydrogen production from microcrystalline cellulose at 37°C and pH 7 in batches, but maximum yields from heat-treated anaerobic digester sludge on 40 g $l^{-1}$ cellulose were just 0.57 mol hydrogen per mol hexose.

#### 1.7.5.3 Hemicellulose

β 1,3, β 1,4-glucans and xylan, a ß-1,4-linked xylose polymer with branches containing substituents such as arabinose, glucose, galactose and glucuronate, are the major components of hemicellulose in the cell walls of forage grasses. It is thought that the hemicellulose is connected to the cellulose via hydrogen bonds, coating and crosslinking the cellulose microfibrils, so that the cellulose/hemicellulose network provides the main structural strength in growing cell walls (Brett and Waldron 1996), making up about 60% of the total grass dry weight (Table 1-7).

There is little information on fermentative hydrogen production from hemicellulose. To date it has only been shown that hydrogen production from simple pentose is possible. Taguchi *et al.* (1995) for example produced 2.06 mol $H_2$ per mol xylose in continuous experiments with a pure culture of *Clostridium sp.* strain No.2 at pH 6, 36 °C and 5h HRT.

Studies by Lee *et al.* (1985b) provide some information on xylanase activity in clostridia. They report that 17 of 20 screened strains of *Clostridium sp.* were able to hydrolyse larch wood xylan. The two strains of *Clostridium acetobutylicum* (NRRL B527 and ATCC 824) they focused on showed maximum xylanase activity at pH 5.8 to 6.0 (pH 4.2 to 6.0 tested) and 65°C (25 to 75°C tested).

#### 1.7.5.4 Other cell wall components in grass

The pectin network is a minor component in the grass cell wall (Hall 2004), accounting for only around 0.5 to 5.5% of the grass dry weight. It consist mainly of galacturonic acid, rhamnose, arabinose and galactose and appears to control the pore size in the cell wall. It is structurally largely independent from the cellulose and hemicellulose network,

but pectins are linked covalently to phenols, cellulose and protein (Brett and Waldron 1996).

Although non-carbohydrate cell wall components are not known to be fermented to hydrogen, they are of importance in that they influence overall grass digestibility. Of the phenolics, ferulic acid may be of major importance to the cell wall structure: it links arabinoxylans through ester links, but it also links lignin to arabinoxylan through a combination of ester- and ether links. Lignin itself is a phenolic polymer, which forms a hydrophobic meshwork in the cell wall. Accounting for around 5 to a maximum of 12% of the total grass dry weight (Table 1-7), it is formed as the last of all networks and cements other wall components in place, thus providing an effective barrier to the penetration of wall-loosening enzymes, nutrients and pathogens (Brett and Waldron 1996). Lignin can not be degraded by hydrogen producers and may inhibit the access of cellulase enzymes to the cellulose (Claassen *et al.* 1999).

#### 1.7.5.5 Pretreatments

To date complex biomass substrates have commonly been pre-treated prior to fermentation, to improve their digestibility. In ethanol fermentation by yeasts for example only simple sugars such as maltose and sucrose are utilised. The main aims of pre-treatments are to remove the lignin and pectin networks and to decrease cellulose crystallinity or even hydrolyse cellulose and hemicellulose to mono/di/oligosaccharides. Resulting hydrolysates should be significantly easier and faster to convert to hydrogen than the original substrate. Taguchi *et al.* (1996) for example report that *Clostridium sp.* strain No.2 was able to produce hydrogen from a cellulose hydrolysate (cellulose pretreated with cellulase) at the short HRT of 6h, pH 6 and 36°C with yields of 4.46 mol hydrogen per mol hexose. However, no explanation was given as to how these high yields, which exceed the theoretical maximum associated with the acetate/butyrate fermentation, could be achieved.

Physical pre-treatment is commercially a very common pre-treatment of lignocellulosic substrates, for example of sugar cane bagasse used for ethanol production. For steam explosion for example, the currently most common pre-treatment, the substrate is treated with high pressure saturated steam followed by rapid pressure reduction, which causes the substrate to decompress explosively (Sun and Cheng 2002). It allows removal

of the lignin and also reduces the cellulose crystallinity and increases the porosity of the substrate. Although this rather energy intensive treatment may not be suitable for grass at laboratory scale, any form of grinding, shredding or cutting should increase grass digestibility.

A range of chemical pre-treatments have also been used on various biomass crops. These generally extract water soluble components, lignin and part of the hemicellulose. Extraction efficiencies of these methods are mostly reported from dried and ground material. For maximum energy yields of fermentative hydrogen production it would be desirable to extract the original wet material directly.

Alkali, for example 1M (Jung *et al.* 1992) or 2M (Hawary *et al.* 2001) NaOH, is commonly used for chemical pre-treatment and has been shown to remove up to 60% lignin (Jung *et al.* 1992) and increase substrate fermentability (Hawary *et al.* 2001) in a range of biomass materials. One disadvantage of alkaline treatment of fermentation substrates is that the alkali will need to be neutralised before inoculum is added, which may result in high salinity of the feedstock. For scale-up $Ca(OH)_2$ (lime) could possibly be used as alternative economically feasible alkali treatment, as for example reported by Kaar and Holtzapple (2000) for treatment of corn stover. Lime is cheaper than NaOH, and could be recovered in scale-up, using established lime kiln technology (as for example for industrial sugarbeet processing).

Alkaline hydrogen peroxide treatment has been used to extract lignin and hemicellulose from wheat straw, alfalfa hay, corn cobs, stalks and husks (Gould 1983), steam-exploded softwood (Yang *et al.* 2002) and wheat bran (Maes and Delcour 2001). For this 1 to 2 % solids were digested in 1 to 2 % hydrogen peroxide at pH 11.5 and 25 to 80°C for 45 min to 24 hours. Different combinations of temperature and duration of incubation were found optimum for different substrates. The method solubilised approximately 90% of the lignin in steam-exploded softwood (Yang *et al.* 2002) and extracted 77% of all arabinose and 65% of all xylose from wheat-bran (Maes and Delcour 2001). Jung *et al.* (1992) compared alkaline hydrogen peroxide treatment with 1M NaOH treatment, both at 39°C for 24 hours, in lucerne, smooth bromegrass and maize. They found that the two treatments removed similar amounts of lignin and neutral sugars from all three plants. Also, *in vitro* xylose digestibility for smooth

bromegrass for example increased from 44% without pre-treatment to 99 and 95% with peroxide and sodium hydroxide pre-treatment respectively.

As well as physical and chemical pre-treatment, enzyme treatment has been applied to many complex carbohydrate substrates. Cellulases are commonly used to hydrolyse the cellulose in a substrate, after lignin and some hemicellulose has been removed in chemical treatment. Kaar and Holtzapple (2000) for example report that 60% cellulose was hydrolysed to glucose when lime pre-treated corn stover was digested with the optimum concentration of 10 Filter Paper Units (FPU) cellulase $g^{-1}$ dry biomass and 28.4 IU β-glucosidase $g^{-1}$ dry biomass at 40°C for 72 hours. Similarly, Yang *et al.* (2002) report that 10 FPU cellulase $g^{-1}$ dry biomass (with added β-glucosidase) achieved complete hydrolysation of cellulose in steam-exploded, alkaline peroxide treated softwood.

No information on the effect of any of these pre-treatments on grass was found in the literature. It can be assumed from the above review of treatments of other biomass that physical pre-treatment would disrupt the cell wall and release water-soluble carbohydrates. Following this, chemical treatment should extract part of lignin and hemicellulose present in the grass, improving access to residual hemicellulose, phenolics and cellulose for any enzymes used in a third treatment. From results by Kaar and Holtzapple (2000) and Yang *et al.* (2002) it can be inferred that optimised cellulase treatment should hydrolyse most of the cellulose. Hemicellulases and ferulic acid esterases may aid hydrolysis of the residual hemicellulose and phenolics.

### *1.7.6 Concentration*

Substrate concentrations used in continuous hydrogen production with mixed microflora are commonly around 10 g $l^{-1}$ (Table 1-8).

***Table 1-8. Substrate concentrations used in continuous hydrogen production with mixed microflora.***

| Substrate | | | Butyrate | Acetate | HRT | pH | Yield[a)] | Reference: |
|---|---|---|---|---|---|---|---|---|
| conc. [g $l^{-1}$] | Type | conversion [%] | conc. [mmol $l^{-1}$] | conc. [mmol $l^{-1}$] | [h] | | | |
| 4.25 | Starch | - | 4.8 | 9.6 | 17.0 | 5.2 | 2.2 | Lay (2000) |
| 7.0 | Glucose | 99 | 10.2 [b)] | 16.7 [b)] | 6.0 | 5.5 | 2.1 | Fang and Liu (2002) |
| 9.8 | Sucrose | 70 | 34 - 57 | 33.3 | 12.0 | 6.8 | 1.8 | Ueno *et al.* (1996) |
| 10.0 | Glucose | - | 26.8 | 17.7 | 3.4 | 6.0 | 1.8 | Van Andel *et al.* (1985) |
| 10.0 | Glucose | 99 | 21.9 | 13.1 | 8.5 | 6.0 | 1.4 | Mizuno *et al.* (2000a) |
| 14.3 | Sucrose | 99 | 58.0 [b)] | 43.3 [b)] | 13.7 | 5.5 | 2.2 | Liu and Fang (2002) |
| 20.0 | Glucose | 19 | 13.6 | 7.8 | 12.3 | 5.0 | 0.4 | Heyndrickx *et al.* (1990) |
| 20.0 | Sucrose | 90 | 10.2-21.6 | 26.7-40.0 | 24.0 | 5.5 | 0.6 | Sung *et al.* (2002)[c)] |
| 20.0 | Sucrose | ~ 99 | 55.4 | 21.0 | 8.0 | 6.7 | 1.7 | Chen *et al.* (2001) |

[a)] units in [mol hydrogen per mol hexose added]
[b)] calculated from % total organic carbon
[c)] fed-batch studies, 3 times a day 1/3 of reactor contents replaced

With respect to the overall energy balance of the process the substrate should be fed in as concentrated form as possible. Srivastava (1987) for example calculate for mesophilic anaerobic digestion that the energy required to heat the feed at 1% w/v TS is about 100% of the total product energy, but only 7-8% at 15 % w/v. As Table 1-8 shows, Heyndrickx *et al.* (1990), Sung *et al.* (2002) and Chen *et al.* (2001) investigated continuous hydrogen production from 20 g $l^{-1}$ substrate. The low substrate conversion rate by Heyndrickx *et al.* (1990) is due to deliberate nitrogen limitation in the culture. Chen *et al.* (2001) and Sung *et al.* (2002) report substrate conversion of 90 and 99% respectively and do not mention any problems with substrate conversion efficiency. However, in the study by Sung *et al.* (2002), a fed-batch experiment in which 3 times a day 1/3 of the reactor contents were replaced with fresh medium, hydrogen yields were

relatively low with 0.6 mol hydrogen produced per mol hexose added. Although Table 1-8 shows that to date higher yields were reported from lower substrate concentrations, results by Chen *et al.* (2001) show that complete substrate conversion and good yields can be achieved from 20 g $l^{-1}$ sucrose substrate. It may therefore be worthwhile to further investigate upper limits to substrate concentration.

In one of few studies on optimum substrate concentration Van Ginkel *et al.* (2001) report from batch experiments (pH 5.5 and 37°C) with a heat shocked mixed culture, that hydrogen yields from sucrose were fairly constant (over 2 mol hydrogen per mol hexose) for sucrose concentrations of 0.5 to 7.5 g $l^{-1}$, whilst substrate concentrations of 15 g $l^{-1}$ or higher resulted in lower hydrogen yields, and yields decreased with increasing substrate concentration in the range 15 to 45 g $l^{-1}$. Products were not analysed and the reason for this decrease in yield was not investigated. Kataoka *et al.* (1997) report from continuous hydrogen production by a pure culture of *Clostridium butyricum* (pH 6.7, 8 h HRT, 30°C) that hydrogen yields decreased from 2.0 - 2.3 mol per mol glucose added at a substrate concentration of 5 g $l^{-1}$ to 1.4 - 2.0 mol per mol glucose at 10 g $l^{-1}$. Since residual glucose concentrations are not mentioned, it is not clear if the substrate was not entirely utilised at 10 g $l^{-1}$ or if the metabolism changed.

Product inhibition is likely to set an upper limit to substrate concentration, since higher substrate concentrations will inevitably result in higher product concentrations (unless conversion rates decrease). The best understood product inhibition mechanism in hydrogen production is high hydrogen partial pressure, which is influenced by a range of parameters and has been discussed in section 1.6.1. Less well investigated but more directly dependent on substrate concentration is product inhibition by volatile fatty acids. It appears that high acid concentrations can cause growth inhibition and/or a change in metabolism from acid to solvent production. As already mentioned in section 1.3.1.3 it is particularly the undissociated form of butyrate that has an inhibitory effect, and the ratio of undissociated/dissociated acid depends on pH. At pH 6.0 for example only 6% of all butyrate would be undissociated, compared to 17% at pH 5.5.

Inhibition of hydrogen production through addition of butyrate has been reported in several studies, but the level of butyrate required to cause a reduction in hydrogen production varies. Some reports suggest that butyrate might have a negative effect on

hydrogen production at undissociated acid concentrations as low as 7 mmol $l^{-1}$. Chin *et al.* (2003) report that at pH 6.0 addition of 114 mmol $l^{-1}$ butyrate, of which only 6.84 mmol $l^{-1}$ is undissociated at pH 6.0, to a batch experiment with a pure culture of *Clostridium acetobutylicum* on glucose (feed concentration not clear and produced butyrate concentration not given) decreased the total amount of hydrogen produced by 30%. Zoetemeyer *et al.* (1982b) report from CSTR experiments at pH 5.5 with activated sludge and 10g $l^{-1}$ glucose that addition of 23 mmol $l^{-1}$ butyrate to a natural concentration of around 17.7 mmol $l^{-1}$ butyrate (giving a total undissociated butyric acid concentration of 7 mmol $l^{-1}$ at pH 5.5) encouraged lactate production and decreased butyrate concentrations to around 13.5 mmol $l^{-1}$. Added butyrate at a concentration of 52 mmol $l^{-1}$ (giving an undissociated butyric acid concentration of 9 mmol $l^{-1}$) caused an increase in residual glucose levels from 0 to 2.5 g $l^{-1}$ (i. e. glucose conversion reduced from 100% to 70%) within 2 days, an increase in lactate production to a concentration of around 27.8 mmol $l^{-1}$ and a decrease in butyrate concentration to around 6.3 mmol $l^{-1}$. Although hydrogen production was not directly measured in this experiment, it can be assumed that the shift from butyrate to lactate production would cause a reduction in hydrogen yields. Bahl *et al.* (1982) report from chemostat culture of a pure strain of *Clostridium acetobutylicum* at pH 4.3 that addition of 20 mmol $l^{-1}$ butyrate caused a metabolic change from acid to solvent production, increasingly so with increasing butyrate concentration in the range of 20 to 60 mmol $l^{-1}$ butyrate. At pH 4.3, 13 mmol $l^{-1}$ of the 20 mmol $l^{-1}$ would be undissociated. However, since observations from lower concentrations of added butyrate were not tested, it is not clear if this is the minimum required for the start of solvent production or if lower concentrations would be sufficient. Addition of acetate in these experiments had no influence on acid or solvent production. Zheng and Yu (2005) report from batch experiments with heat treated anaerobic digester sludge on 10 g $l^{-1}$ glucose, that hydrogen yields were strongly reduced from 1.75 to 0.32 mol per mol hexose converted when 285 mmol $l^{-1}$ butyrate was added at pH 5.75 to 6.0, which gives an undissociated butyric acid concentration of approximately 17 mmol $l^{-1}$.

In contrast to these findings, including their own findings in the same study as described above, Zoetemeyer *et al.* (1982b) report that at pH 6.0 90 to 97% of glucose could be converted to acids, gas and biomass when glucose concentrations were 2.5 to 75 g $l^{-1}$.

Feed concentration of 75 g $l^{-1}$ (the highest they tested) resulted in butyrate concentration of 273 mmol $l^{-1}$, giving at pH 6.0 an undissociated butyric acid concentration of 16 mmol $l^{-1}$. Judging by their results with added butyrate, this should cause product inhibition, but they achieved hydrogen yields of 1.43 mol per mol glucose.

The level of undissociated butyric acid required to negatively affect hydrogen production may also depend on other factors, such as species, substrate or hydraulic retention times. Fond *et al.* (1985) for example report from mesophilic fed batch studies on glucose that the butyric and acetic acid concentrations required to change the dominant metabolism of *Clostridium acetobutylicum* from acid to solvent production depend on the rate of glucose uptake and the culture pH. The studies showed that at pH 4.8 acid concentrations of 40 to 50 mmol $l^{-1}$ are sufficient to start solvent production when glucose was fed at a rate of 7 g $l^{-1}$ $day^{-1}$, but 110 to 160 mmol $l^{-1}$ acid was required when glucose was fed at a rate of 2.8 g $l^{-1}$ $day^{-1}$. The authors explain the dependence of the critical acid levels on rate of metabolism with the difference in intra- and extracellular acid concentrations: they assume that higher feed rates create a faster carbon flow through the cell, causing higher intra- than extracellular acid concentrations. At lower feed rate, the equilibrium between intra- and extracellular acid concentrations would be better maintained.

In summary it can be concluded that little is known about optimum substrate concentrations for hydrogen production. Product inhibition has been observed, but at various product concentrations, and there is only little and patchy knowledge on the effect of other operating parameters on optimum substrate concentrations. Since an increase in substrate concentrations would increase the efficiency of the system significantly, further research is required.

### *1.7.7 Nutrients*

Little appears to be known about the nutrient requirements of hydrogen producers, and the composition of nutrient solutions used in experiments with mixed cultures varies widely as the examples in Table 1-9 show. It can be assumed that for most studies nutrient concentrations were selected to be well in excess to ensure that the environment is carbon limited, as for example stated in Zoetemeyer *et al.* (1982a).

***Table 1-9. Element concentrations [mg $l^{-1}$] of defined media in continuous hydrogen production with mixed cultures***

| Element | Fang and Liu (2002) | Lin and Chang (1999) | Mizuno *et al.* (2000a) | Zoetemeyer, Arnoldy *et al.* (1982) |
|---|---|---|---|---|
| N | 131 | 929 | 680 | 351 |
| P | 102 | 22 | 45 | 78 |
| K | 112 | 56 | 113 | 97 |
| S | 42 | 5.5 | 0.6 | 12.8 |
| Mg | 32 | 13 | 32 | 3.6 |
| Fe | 17 | 5.0 | 1.0 | 2.4 |
| Ni | 12 | - | 0.13 | - |
| Ca | 14 | - | - | 0.20 |
| B | 0.4 | - | 0.07 | - |
| Mo | 0.01 | - | 0.20 | - |
| Zn | 11 | - | 0.24 | 0.40 |
| Co | 6.2 | 0.04 | 0.74 | - |
| Cu | 3.7 | 1.3 | - | - |
| Mn | 8.3 | - | 0.69 | - |
| I | - | - | 1.91 | - |
| hexose | 7000 | 20000 | 10000 | 10000 |
| COD:N | 57:1 | 23:1 | 16:1 | 30:1 |
| COD:P | 73:1 | 970:1 | 240:1 | 140:1 |

Although there are reports of nitrogen fixing clostridia, for example *Clostridium arcticum, Clostridium butyricum* and *Clostridium pasteurianum* (Ljungdahl *et al.* 1989), and there are no studies on optimum nitrogen concentrations, it seems to be taken for granted that hydrogen production from most carbohydrate rich substrates requires addition of nitrogen. Table 1-9 shows that COD:N ratios of 16 to 57:1 have been applied. Equally, there is little information about the form in which the nitrogen needs to be provided. A number of saccharolytic butyric acid producing clostridia are known to be able to use inorganic N as their sole nitrogen source (Woods and Jones 1986), and several studies of continuous hydrogen production report yields of more than 1 mol hydrogen per mol hexose with addition of mineral nutrients only (Mizuno *et al.* 2000a; Lay 2000; Fang and Liu 2002).

However, addition of an organic nitrogen source may increase yields. Yokoi *et al.* (1998b) state that addition of yeast extract with an optimum concentration of 0.5 g $l^{-1}$ to the mineral medium was essential for hydrogen production from starch by *Clostridium butyricum* and *Rhodobacter sp. M-19* in batch studies. Further experiments showed that

a mixed culture of *Clostridium butyricum* and *E. aerogenes* could not produce hydrogen from starch residue in fed-batch experiments when inorganic N or urea was the sole nitrogen source. Addition of 0.1% polypeptone as N source gave maximum hydrogen yields of 2.4 mol hydrogen per mol hexose from this culture (Yokoi *et al.* 2001). In a later study Yokoi *et al.* (2002) replaced polypeptone successfully with corn steep liquor, a liquid waste from corn starch manufacturing which is considerably cheaper than polypeptone. Ueno *et al.* (2001) made similar observations when studying hydrogen production from cellulose by a mixed culture in thermophilic chemostat experiments. When mineral nutrients including 0.5 g $l^{-1}$ $NH_4Cl$ as nitrogen source were added to 5 g $l^{-1}$ pure cellulose substrate, a yield of 1 mol hydrogen per mol hexose was obtained. The COD:N ratio in this medium was 41:1, which was within the range reported in other studies (Table 1-9). However, the hydrogen yield could be increased from 1 to 2 mol hydrogen per mol hexose when 5 g $l^{-1}$ peptone was added. Analysis of the microbial community showed that this also allowed growth of a wider variety of species.

Phosphate has also been added in all experiments where successful hydrogen production was reported, but little information is available on optimum phosphate concentrations for hydrogen production. Lin and Lay (2004) report from batch experiments with acclimated anaerobic sludge on 17.8 g $l^{-1}$ sucrose that addition of 600 mg $l^{-1}$ $Na_2HPO_4$ gave maximum hydrogen production. This equals a COD:P ratio of 145:1, which is similar to that of 140:1 used by Zoetemeyer *et al.* (1982a) (Table 1-8). Lin and Lay (2004) do not give actual yields but state that a 30% lower or higher $Na_2HPO_4$ concentration causes a 40% decrease in hydrogen production. Phosphate limitation to COD:P of 34000:1 (30 g $l^{-1}$ glucose, 0.03 mM phosphate) was reported to inhibit cellulolytic enzyme activity in strains of *Clostridium acetobutylicum* (Lee *et al.* 1985a). However, this phosphate concentration is magnitudes lower than the concentrations commonly used (see Table 1-9) and therefore has little value for determination of optimum phosphate concentrations. As Table 1-9 shows COD:P ratios in hydrogen producing cultures vary widely from 70 to 970:1 for pure carbohydrate feed. In solvent production phosphate addition has also been found essential for more complex feedstocks such as beet and invert molasses, which can be expected to contain some phosphate already (Biebl 1999).

Interestingly, Heyndrickx *et al.* (1990) reports very good hydrogen yields from continuous experiments with *Clostridium butyricum* at pH 7.0 when phosphate was limited to 0.5 mM, giving a COD:P ratio of 4130:1 for 60 g $l^{-1}$ glucose. Only 25 g $l^{-1}$ glucose were converted, presumably because substrate conversion was restricted by phosphate limitation, but hydrogen yields of 2.22 mol hydrogen per mol glucose converted were achieved. The authors explain these high yields with the observation that phosphate limitation increased glucose conversion to fermentation products and decreased that to biomass.

Although Ren *et al.* (1995) report hydrogen production from sugar or cornstarch with addition of N and P salts in the ratio of COD:N:P = 800-1000:5:1 only, most studies have used more complex nutrient solutions. Information on how essential these nutrients actually are is rare and scattered. However, the standard methods for assessment of biodegradability in anaerobic digester sludge use a medium containing N, P, K, Ca, Mg, Fe, B, Mn, Co, Zn, Ni, Cu and Na (HMSO 1988), which suggests these elements must be essential for growth of a mixed anaerobic culture. Bahl and Gottschalk (1984) showed that limitation of magnesium to 0.075 mmol $l^{-1}$ reduced bacterial growth but promoted acetate/butyrate production. Lin and Lay (2005) reported that the Mg concentration was particularly influencial on hydrogen production in experiments where the importance of 13 nutrients in 3 concentrations (individually varied) was investigated in batch experiments with acclimated anaerobic sludge on 17.8 g $l^{-1}$ sucrose using a fractional design method. The optimum concentration was found to be 1.26 mmol $l^{-1}$ Mg, and according to the applied statistical model a 30% increase or decrease in Mg concentration caused a 40% decrease in hydrogen production.

The hydrogen forming hydrogenase in clostridia as well as the hydrogen carrier ferredoxin, which are both vital to hydrogen production (section 1.3.1), are iron sulphur proteins (Mitchell 2001). Ferredoxin contains 2 $Fe_4S_4$ clusters (Moat *et al.* 2002). The hydrogen producing hydrogenase contains 5 [Fe-S] clusters and has an iron content of 20.1±0.7 g $mol^{-1}$ protein and a sulphur content of 17.8±1.2 g $mol^{-1}$ protein (Peters *et al.* 1998). It can therefore be assumed that addition of iron and sulphur to the substrate are vital to hydrogen production. However, only few publications investigated the actual effect of iron or sulphur concentrations on hydrogen or butyrate production. Chen *et al.* (In Press) showed in batch experiments that addition of iron was essential for hydrogen

production from a pure strain of *Clostridium butyricum* on 17.8 g $l^{-1}$ sucrose. Hydrogen yields in an iron-free medium were negligible, (max. ~ 0.04 mol hydrogen per mol hexose added), whilst hydrogen yields of 1.5 mol hydrogen per mol hexose converted were achieved when 10 mg $l^{-1}$ $Fe_2SO_4 \cdot 7\ H_2O$ was added. Although this shows that iron is important to hydrogen production, iron concentration in the medium was not optimised.

Schoenheit *et al.* (1979) showed that ferredoxin is synthesized at a minimum iron concentration of 1 µmol $l^{-1}$ in *Clostridium pasteurianum*. At lower iron concentrations ferredoxin is degraded to form other enzymes. Therefore hydrogen production should be reduced by iron limitation, even though growth might not be reduced, since ferredoxin is not essential for growth. This would explain observations from continuous experiments with *Clostridium pasteurianum* on glucose by Dabrock *et al.* (1992), in which iron concentrations below 5.7 µmol $l^{-1}$ (0.3 mg $l^{-1}$) favoured lactate over butyrate production.

Batch studies with mixed cultures on sucrose by Lee *et al.* (2001) and Zhang *et al.* (2005) showed optimum concentrations of 800 mg $FeCl_2$ $l^{-1}$ and 800-1600 mg $FeSO_4$ $l^{-1}$ respectively, giving a yield of 1.0 and 1.4 mol hydrogen per mol hexose respectively. These "optimum" iron concentrations however are around 2 magnitudes higher than concentrations used in continuous hydrogen production with yields over 2 mol hydrogen per mol hexose (for example Fang and Liu 2002), which questions the significance of this result for hydrogen production with mixed cultures.

No direct information is available on the effect of sulphur limitation on a hydrogen producing culture. Bahl and Gottschalk (1984) report that at pH 5.0 or above, lactate production exceeded acetate/butyrate production when *Clostridium acetobutylicum* was grown continuously in sulphate limitation of 0.20 mmol $l^{-1}$.

# 2 Materials and Methods

## *2.1 Experimental apparatus*

### *2.1.1 Reactors*

Two anaerobic continuously stirred tank reactors (CSTR) with working volumes of 2.3 l (reactor A) and 9.5 l (reactor B) were used. A typical schematic of the experimental apparatus is given in Figure 2-1 and photos of reactors A and B can be found in the appendix.

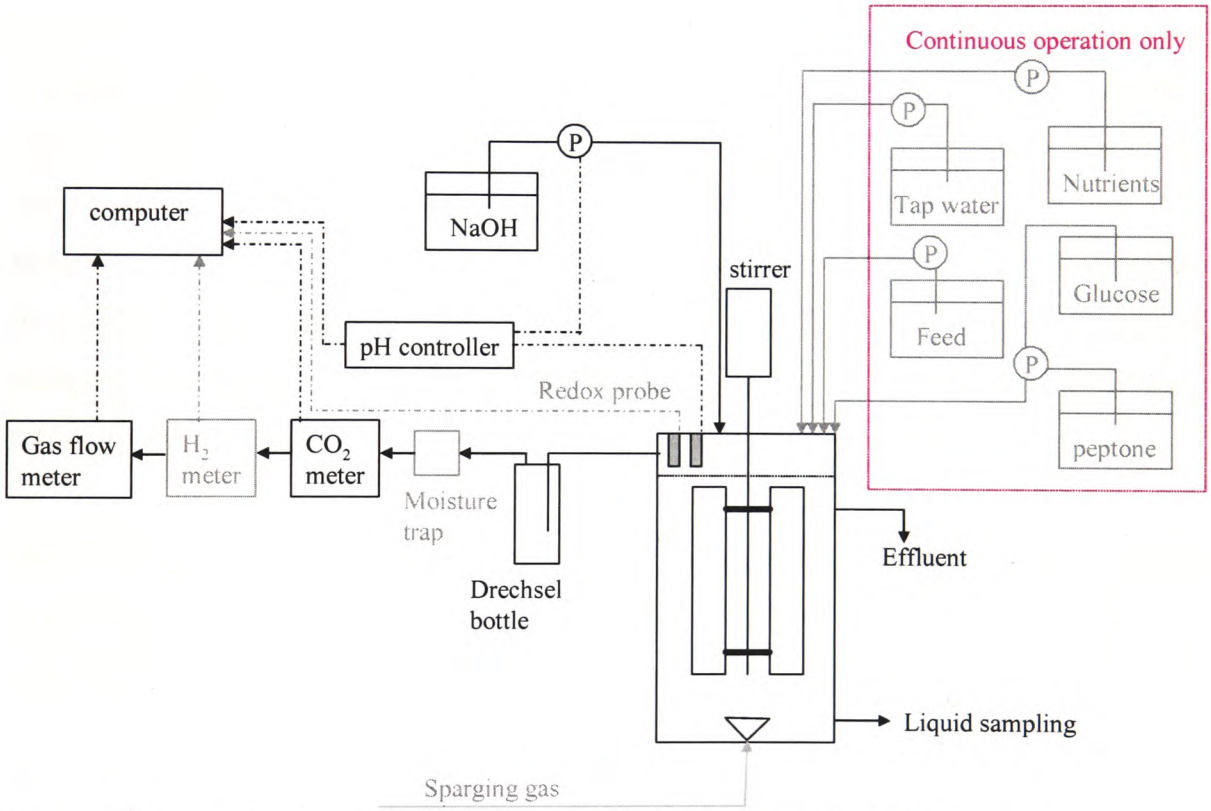



***Figure 2-1. Schematic of experimental apparatus. Parts in grey: not used in all experiments.***

Both reactors were constructed from Perspex at the workshops of the University of Glamorgan and were of cylindrical shape with a detachable lid. The lids contained a number of ports for feed, nutrient, water and alkali input, fitting of pH and redox probes and gas output. All liquid ingredients which had to be added continuously were pumped from the storage reservoir through silicon tubing to the respective port in the reactor lid with Watson Marlow 505U/RL or 505S pumps fitted with Marprene tubing (Watson

Marlow, Falmouth, UK). The liquid level in the reactor was controlled by the shape and position of the pipe through which the effluent exited the reactor. Originally a U-bend as shown in Figure 2-2a was fitted to all reactors and used for all experiments with wheat starch. However, the fibre in the sugarbeet caused the pipe to block, therefore it was shortened as shown in Figure 2-2b for experiments with sucrose/sugarbeet and grass.

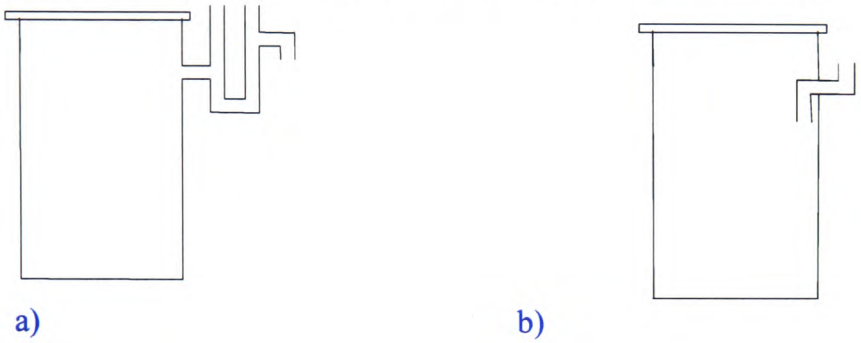



*Figure 2-2. Schematic of effluent pipes used.*

A liquid sampling port was fitted in the reactor wall close to the bottom of each reactor. Liquid samples (10 to 40 ml) were taken with a 50 ml plastic syringe fitted to the liquid sampling port, which was clamped off with a Hoffman clip when not in use. For sampling, reactor contents were repeatedly drawn into the syringe and re-injected into the reactor before taking of the sample to ensure it was representative of reactor contents. In intensively monitored batch studies, the volume of sample taken was replaced with tap water.

Gas from the reactor headspace was passed through a Drechsel bottle containing approximately 30 ml tap water (or saturated $Cu_2SO_4$ solution, see section 2.1.3.2), a silica moisture trap, carbon dioxide monitor, hydrogen sensor and gas flow meter. For experiments with starch and batch studies with sucrose gas was sampled at a T-junction in the gas tubing between the reactor and the Drechsel bottle. Since the moisture in the gas samples damaged the thermal conductivity detector used for offline hydrogen determination, for continuous operation on sucrose and experiments with grass gas samples were taken between the moisture trap and the carbon dioxide meter. Reactor A had a headspace of approximately 600 ml to this sampling point. For reactor B the headspace to the same point was estimated at ca. 1500 ml.

The reactors had a stirrer consisting of a stainless steel rod and 2 vertical Perspex (reactor A) or aluminium (reactor B) blades. The stirrers were rotated by an adjustable electric stirrer head (Heidolph Instruments, Schwabach, Germany) at 90 –110 rpm.

For sparging, which was only implemented in reactor A, nitrogen was passed from a high pressure cylinder (Messer, Cardiff, UK) through gas tight tubing via 2 control valves and a gas flow meter to a port at the bottom of the reactor. On the inside of the reactor a sinter stick functioning as gas disperser was attached to the port at the reactor bottom. Details on the sintersticks used for different experiments are given in section 2.7.1.2.

All gases used for sparging, instrument calibration and operation were GC grade and obtained from Messer (Cardiff, UK) unless stated otherwise.

### *2.1.2 Control of environmental conditions*

#### 2.1.2.1 Temperature control

The temperature in the reactors was controlled to an accuracy of ± 0.3°C by a water jacket connected to a flow heater (Grant Instruments, Cambridge, UK). The temperature was monitored by the online pH controller and was checked daily, but data was not recorded. For reactor A the water jacket consisted of silicon tubing coiled around the reactor, reactor B was double walled.

#### 2.1.2.2 pH control

The operating pH was measured with a combination polymer pH electrode type InPro4010/120/PT1000 or HA405-DXK-S8/120 (Mettler Toledo, Leicester, UK), connected to a pH meter/controller from Mettler Toledo (Leicester, UK) or ABB Kent Taylor (St. Neots, UK). Immediately before each experiment the instrument was calibrated with 2 NIST (National Institute of Standards and Technology) traceable standards at pH 4 and 7. The pH meter was directly connected to the online data acquisition system described in section 2.1.3.5. The set-points on the controller were chosen to maintain the reactor pH within 0.05 pH units of the chosen operating pH by activating a Watson Marlow (Falmouth, UK) 505U/RL pump when the lower threshold

value was reached, adding 1 M NaOH to the reactor until the upper threshold value was reached.

### *2.1.3 Online monitoring*

The ingoing and outgoing gas flow, pressure of the ingoing gas, hydrogen and carbon dioxide content of the outgoing gas, redox potential and pH were monitored online The same online monitoring system was used for both reactors A and B.

#### 2.1.3.1 Gas flow

The ingoing gas flow was measured with an in-line volumetric flow meter from Cole Parmer (London, UK), which operates on the principle of measurement of differential pressure across a Laminar Flow Element. The meter was chosen because it is user-programmable to read flowrates of several gases including carbon dioxide and $N_2$. However, it was observed that after 3 months the meter, which can be tared to zero manually, developed a tendency to drift, up to 10 ml $min^{-1}$. This could have been caused by passing moist gas through the instrument. Therefore the meter readings were only used as indicator for short-term relative changes in sparging rate, but not for calculation of total hydrogen production.

The exiting gas rate was measured with 3 different gas meters depending on flow rate. For flow up to 16 ml $min^{-1}$ a low flow gas meter type LFM300/1 (Alexander Wright Ltd, London, UK) was used. The meter was calibrated with standards traceable to National Physical Laboratory Standards by Zeal Ltd. (London, UK) for the range 1.7 to 16 ml $min^{-1}$ to within ±0.25% of the actual flow-rate over the full range. For experiments where the gas flow rate exceeded this range, a low flow gas meter type LFM300 (Alexander Wright Ltd, London, UK) or a volumetric flow meter type ADM2000 from Agilent (Placerville, USA) was used. The LFM300 was also calibrated with standards traceable to National Physical Laboratory Standards by Zeal Ltd. (London, UK), for up to 67 ml $min^{-1}$ with accuracy of ±0.25% throughout the calibration range. The ADM2000 was calibrated by Agilent (Placerville, US) with NIST traceable standards for up to 1000 ml $min^{-1}$ to an absolute accuracy of 3% throughout the range.

#### 2.1.3.2 Hydrogen content

The hydrogen content of the exiting gas was determined with a hydrogen sensor from H2scan (Valencia, California, USA). A photo of the sensor is given in the appendix. The sensor contains a thin film of palladium/nickel deposited on a silicon substrate. The lattice inherent in the Pd/Ni absorbs hydrogen molecules. The resistance of the Pd/Ni increases in direct correlation to the amount of hydrogen present. The H2SCAN$^{TM}$ system connected to the sensor converts this resistance to a voltage output, which is directly proportional to the hydrogen concentration and can be read by LabVIEW$^{TM}$. The system was custom calibrated by the supplier to convert hydrogen concentrations in the range of 0 to 100% to 0 to 5 V analogue output. It operates best at flow rates of 230 to 1410 ml $min^{-1}$. At smaller flow rates the response time increases by a few seconds. The sensor is hydrogen specific, so there is no interference by any other element or compound gas. It operates at 0 to 40°C and 0 to 95% relative humidity and has a heating system which keeps the sensor substrate at a constant temperature, thus the sensor output is unaffected by ambient temperature. Since the aluminium case which protects the sensor can be corroded by $H_2S$, the tap water in the Drechsel bottle before the moisture trap was replaced with saturated $Cu_2SO_4$ solution. Sensor readings were tested with 0 and 100% hydrogen standards and found accurate to within 0.1 and 1% respectively. Hydrogen was measured on-line in experiments St8, St9 and Gr1, Gr2 and Gr3.

The on- and offline data for hydrogen content obtained from experiments (Gr1 to Gr3, St8 and St9, see sections 2.7.1.2 and 2.7.3) is compared in Figure 2-3.

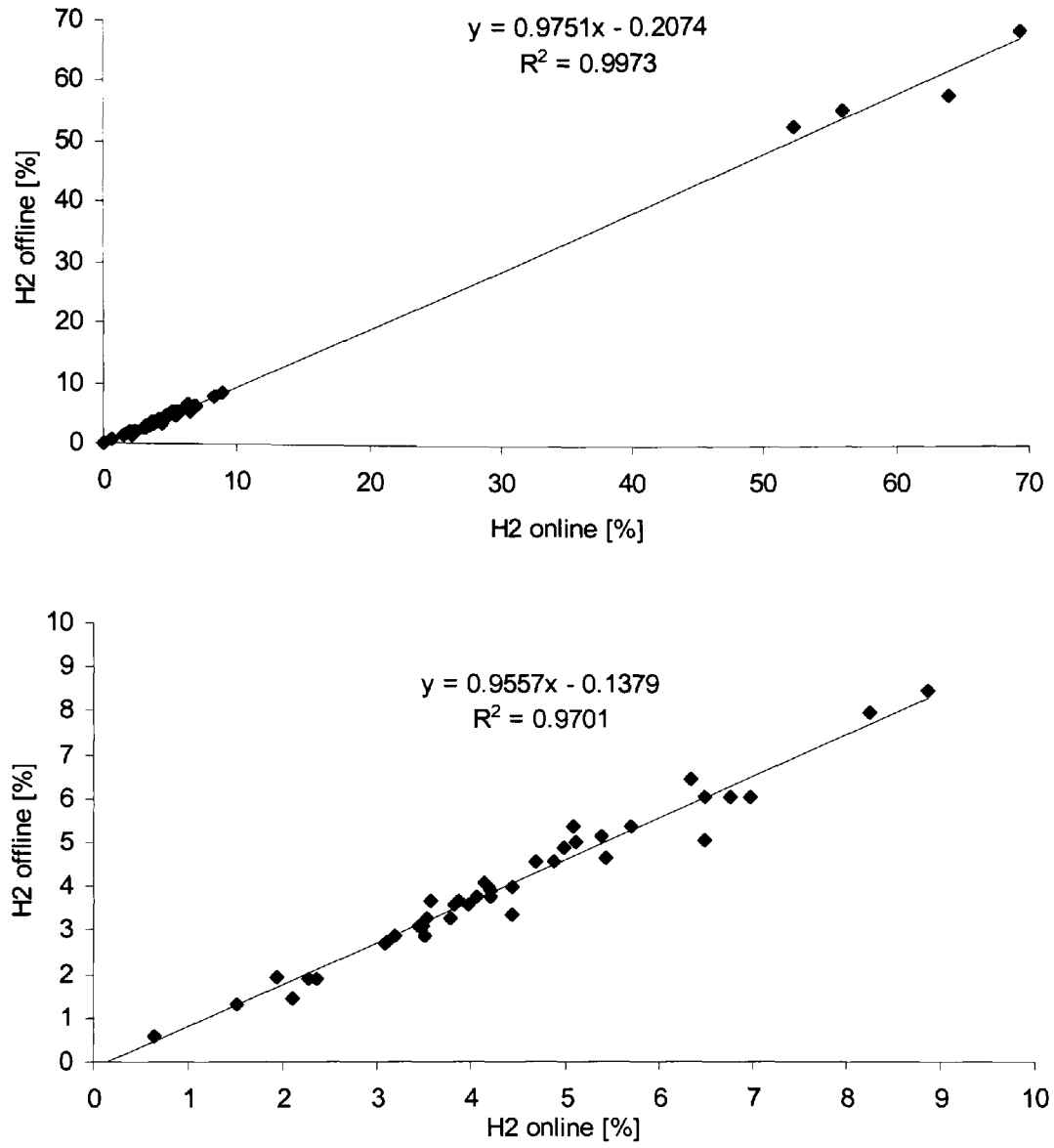



***Figure 2-3. Comparison of offline (GC) and online ($H_2$ sensor) hydrogen content measurements. a) in the range 0-70 %, including all datapoints of experiments St8, St9, Gr1, Gr2 and Gr3. b) in the range 0-10%, i. e. excluding datapoints of experiment Gr1.***

Figure 2-3 shows that online measurements with the $H_2$ sensor and offline measurements with the gas chromatograph are linearly correlated, giving an $R^2$ value of >0.97. This suggests that the hydrogen sensor, which has not previously been used for monitoring of biohydrogen production, is a reliable method of on-line hydrogen percentage measurements.

#### 2.1.3.3 Carbon dioxide content

The carbon dioxide content of the produced gas was measured with an instrument holding a Gascard II infra-red gas sensor from Edinburgh Sensors Ltd (Livingston, UK). The instrument measures the amount of infra-red energy absorbed by the gas sample and thus quantifies the number of molecules. The readings are automatically temperature and pressure corrected, and the instrument is calibrated by the supplier in the range of 0 to 100% $CO_2$ to an accuracy of within 2% of the full range. Similar to the hydrogen sensor, the minimum recommended gas flow rate through the instrument is 200 ml $min^{-1}$. Lower flow rates are expected to cause a lag in response time, which was found to be less than 1 minute for flow rates of 10-20 ml $min^{-1}$ when tested with a change from pure nitrogen to pure carbon dioxide (both gases from Messer, Cardiff, UK). The calibration was checked regularly with carbon dioxide standards (100 % from Messer, Cardiff, UK, 40% and 1% from BOC, Guildford, UK). The sensor is insensitive to relative humidity, but -as the hydrogen sensor- may be damaged by condensation.

#### 2.1.3.4 Redox potential

The redox potential was measured with a combination redox electrode type Pt4805-DXK-S8/120 from Mettler Toledo (Leicester, UK). The probe has a platinum redox sensor, Xerolyt electrolyte (a polymer containing KCl), a ceramic diaphragm and an Ag/AgCl reference system. According to the supplier, the probe covers a redox range of approximately -1200 mV to 1200 mV. The electrode can not be calibrated, but was tested before and after each experiment with NIST traceable standards at 220 mV (pH 7, 25°C) and 468 mV (pH 0.1, 25°C), also from Mettler Toledo (Leicester, UK). If readings did not agree with the buffer value to within ±20 mV (as recommended by the supplier), the probe was cleaned as instructed by the supplier. The redox potential was measured in experiments St8 and St9, Su8, Su9 and Su10 and Gr1, Gr2 and Gr3. The recorded redox value is the actual voltage created by the probe and is reported as measured by the instrument (against Ag/AgCl reference system) at the actual operating pH and temperature.

#### 2.1.3.5 LabVIEW™ data acquisition

For online monitoring, data were logged to a Viglen Pentium III computer. The PC was fitted with a data acquisition system from LabVIEW™ (Newbury, UK), consisting of a PCI-6035E data acquisition card, NI-DAQ driver software and a LabVIEW™ 6 full

development system for Windows. All online monitoring instruments but the ADM2000 gas flow meter had an analogue voltage output option and were connected to the data acquisition card via a CB-68LP I/O connector block. In experiments where hydrogen was not measured online, all analogue instruments were grounded as referenced single ended. When the hydrogen sensor was added for experiments on grass and St8 and St9, this caused strong instrument interference with pH and redox measurements. For these experiments pH and redox sensors were therefore grounded as differential, whilst all other instruments were left as referenced single ended. In experiments where only instruments with analogue output were used, the data were logged every 10 seconds and displayed on screen in the form of graphs. The data were averaged in LabVIEW™ over 5 minutes and then written to file. The ADM2000 gas flow meter is an RS232 output device and had to be connected to LabVIEW™ via the serial port. When in use, the rate at which the digital data were delivered to the computer dictated the frequency of data-logging, so that data were written to file every 5 to 7 minutes.

## *2.2 Off-line analysis*

### *2.2.1 Hydrogen*

The hydrogen content of the biogas was determined off-line with a Star 3400CX gas chromatograph (GC) from Varian Ltd. (Walton-upon-Thames, UK). The GC was fitted with a 4 m stainless steel column packed with Porapack N 80-100 (Supelco Ltd, Poole, Dorset, UK) and a thermal conductivity detector. The injector was set to 80°C, the column to 70°C and the detector to 200°C. The sample run time was set to 3 min. GC grade nitrogen (Messer, Cardiff, UK) at a flow rate of 23 ml $min^{-1}$ was used as the carrier gas.

The GC was calibrated daily with a 3 point calibration of volumes 200, 600 and 1000 µl, using 100% hydrogen. A five point calibration in the same range was performed every time the GC temperatures or gas pressure had been changed. The $R^2$ value of the calibration curve always exceeded 0.990.

Biogas samples were drawn from the reactor port with a glass syringe fitted with a two-way valve. For experiments without sparging, when the hydrogen content of the produced gas was approximately 50%, a 1 ml sample was injected with a 2 ml syringe. For experiments with sparging, when the hydrogen content of the produced gas was

approximately 5%, a 4 ml sample was injected with a 5 ml syringe. For sampling the syringe was connected to the sample port, the Hoffman clip and syringe valve opened, and the syringe filled once, disconnected, valve and clip closed, and the sample discarded to ensure that the analysed sample is representative of the biogas and not mixed with air. The filling of the syringe was then repeated, but the piston drawn repeatedly to mix the gas in the gas line thoroughly with the gas in the syringe, before the syringe was completely filled with sample, valve and clip closed, and the syringe disconnected from the port. Just before injection the needle was put on and approximately 1 ml of sample pushed out. Since the sample was injected directly without a sample loop, the sample volume was recorded to an accuracy of 0.1 ml for the percentage calculation. For a 1 ml sample, this limited the accuracy of the method to approximately +/- 5%.

### *2.2.2 Carbon dioxide and methane*

The carbon dioxide and methane content of the produced gas was measured off-line with the same gas chromatograph as used for hydrogen, to check the accuracy of the online carbon dioxide measurements and the presence of methane when carbon dioxide and hydrogen in the produced biogas did not add up to 100%. The column used was a 2 m stainless steel column packed with Porapak Q 50 - 80 (Supelco Ltd, Poole, Dorset, UK) at 60°C. The injection port was heated to 110°C and had a sample loop fitted. The carrier gas was helium (GC grade from Messer, Cardiff, UK) at a flow rate of 23 ml $min^{-1}$. The gas was quantified with the same TCD at 200°C as used for hydrogen quantification. Samples were taken with the same procedure as described for hydrogen analysis, but with a 10 ml plastic syringe with a 3 way valve, so that the syringe did not need to be removed from the sampling port for discarding of the first gas sample drawn. The GC was calibrated to within 5% on day of use with a single point calibration using a 60:40 % mix (accuracy of mix guaranteed to 1 decimal place) of $CH_4$ and $CO_2$ (BOC, Guildford, UK).

### *2.2.3 Volatile fatty acids*

Volatile fatty acids were determined with a head-space gas chromatograph (HS-GC) as described in Cruwys *et al.* (2002). The instrument used was a HS 40XL automatic headspace sampler connected to an Autosystem XL GC system, both from Perkin-Elmer (Beaconsfield, UK), which was calibrated to determine concentrations of acetic,

propionic, isobutyric, n-butyric, isovaleric and n-valeric acids in the range of 0 to 1000 mg $l^{-1}$ (9 point calibration). As stated in Cruwys *et al.* (2002), who used the same instrument, the detection limit was below 4 mg $l^{-1}$ for all acids, and analysis of ten replicate wastewater samples gave standard deviations of 3.9 to 6.5%.

Samples were prepared by pipetting 1 ml sample, 1 ml deionised water, 1 ml $NaHSO_4$ (62% w/v) and 0.1 ml 2-ethylbutyric acid (1800 mg $l^{-1}$, stored at 4°C) as internal standard into a standard 22.3 ml vial, fitted with PTBE septum and patented closure (Perkin-Elmer, Beaconsfield, UK).

In the head-space unit vials were thermostatted for 30 min and then pressurised for 3 min. The sample injection period was 0.1 min. Each sample was followed by 2 washes to minimise carry-over of sample. The injection port was maintained at 200°C and split flow of 5.0 ml $min^{-1}$. The column used was a free fatty acid phase fused-silica capillary column (30m × 0.25 mm I. D., film thickness 0.25 μm) from Perkin-Elmer. Initial column temperature was 60°C, which was increased at 10°C $min^{-1}$ to 200°C, where it was held for 1 min. The carrier gas was nitrogen at a pressure of 14 psi. The detector was a flame ionisation detector (FID) at 250°C.

### *2.2.4 Solvents*

Acetone, ethanol and butanol concentration was determined in all experiments but St2 with a Star 3400 GX gas chromatograph from Varian Ltd. (Walton-upon-Thames, UK) fitted with a 8200 CX autosampler, a 2 m Carbowax column filled with Carbopack 80/120 (Supelco Ltd, Poole, Dorset, UK) and a FID. Injection port and FID temperatures were 120°C and 220°C respectively. The initial column temperature was 65°C, where it was held for 5 min and then increased by 4°C $min^{-1}$ to 200 °C. The carrier gas was GC grade nitrogen from Messer (Cardiff, UK) at a flow rate of 20 ml $min^{-1}$. The runtime for each sample was 32 min.

The GC was calibrated for acetone, ethanol and butanol with a 6 point calibration in the range of 25 to 1000 mg $l^{-1}$. The detection limit was below 25 mg $l^{-1}$. Hexanol was used as internal standard. To avoid blocking of the autosampler needle samples were either centrifuged for 3 min at 3000 rpm or filtered through a Whatmann GF/C filter. For use

in the autosampler 600 µl clear sample or standard and 600 µl of internal standard (500mg $l^{-1}$ hexanol in deionised water acidified with phosphoric acid) were pipetted into a 12mm × 32 mm screw cap vial (Alltech Associates, Stamford, UK) and sealed with a TFE/silicon lined septum and lid. Triplicate samples had a standard deviation of up to 10%.

### *2.2.5 Lactate and formate*

Lactate and formate were analysed for experiments St8, St9, Su9, Su10 and Gr1 to Gr3 with a high performance liquid chromatograph (Dionex, Camberley, UK). The instrument was fitted with a GP40 gradient pump, an IONPAC ICE-AS6 column for organic acids, anion-ICE micromembrane suppressor (AMMS-ICE II) and CD20 conductivity detector (all from Dionex, Camberley, UK). 0.4 mM heptafluorobutyric acid at a flow rate of 1.0 ml $min^{-1}$ was used as eluent. The regenerant for the suppressor was 5 mM tetrabutylammonium hydroxide solution at a flow rate of 2.5 to 3.0 ml $min^{-1}$. The injection port was fitted with a 50µl sample loop. On the day of use the instrument was calibrated with a standard of 10 mg $l^{-1}$ lactate and 10 mg $l^{-1}$ formate in deionised water. Readings for standards prior to calibration varied from day to day by a maximum of 5%. Reactor samples were filtered through a Whatmann GF/C filter or centrifuged at 3000 rpm, then diluted 1:100 or 1:1000 and injected with a 2 ml plastic syringe through a 1.0 µm syringe filter (GelmanSciences, Ann Arbor, USA).

### *2.2.6 Solids*

Total solids were determined by drying of a sample of known volume at 103 to 105°C to constant weight (APHA 1989). For this 3 100ml Pyrex beakers (for 3 replicate samples) were dried in an electric furnace (Carbolite, Sheffield, UK) at 500±50° for at least 1 h. The beakers were then cooled to room temperature in a desiccator and their weight determined to 4 decimal places. Depending on the expected solids content of the sample, a known volume (10 to 30 ml for liquid samples) or mass (5 to 15 g) of well mixed sample was placed in each of the weighed beakers and then dried to constant weight in a Hot Box convection oven (Gallenkamp, Leicester, UK). Before weighing to 4 decimal places the dried sample was cooled in a desiccator to room temperature.

The total solids were calculated as in equation 19.

$$TS\ [g\ l^{-1}] = (A - B) * 1000 / V \quad or \quad TS\ [g\ kg^{-1}] = (A - B) * 1000 / W \qquad \textit{Equation 19}$$

Where:

TS: total solids

A: weight of beaker and dried sample [g]

B: weight of beaker [g]

V: sample volume [ml]

W: sample weight [g]

After total solids analysis the samples were incinerated in an electric furnace (Carbolite Ltd, Sheffield, UK) at 500±50°C for at least 1 hour for determination of volatile solids. After cooling to room temperature in a desiccator the incinerated samples were weighed to 4 decimal places. The fraction of sample lost between heating to 105°C and 500°C is defined as volatile solids (which may include some structural water) and is calculated as in equation 20.

$$VS\ [g\ l^{-1}] = TS - ((A-B) * 1000 / V)$$

$$or\ VS\ [g\ kg^{-1}] = TS - ((A-B) * 1000 / W) \qquad \textit{Equation 20}$$

Where:

VS: volatile solids

TS: total solids [g $l^{-1}$]

A: weight of beaker and incinerated sample [g]

B: weight of beaker [g]

V: sample volume [ml]

W: sample weight [g]

Suspended solids were determined by drying to constant weight the filter residue of a known volume of sample at 103 to 105°C. Suspended solids was defined as the fraction of solids that can not pass through a Whatman GF/C filter (1.2 μm pore size). For

preparation 3 100 ml pyrex beakers (for 3 replicate samples) containing 1 Whatman GF/C filter each were dried in an electric furnace at 500 ± 50°C for at least 1 hour. The beakers were then cooled to room temperature in a desiccator and weighed to 4 decimal places. The filter paper was placed into a Hoffman filter unit, to which a vacuum was applied via a Buchner flask. A known volume of well mixed sample (5 to 15 ml depending on solids content) was then pipetted onto each filter. Once the filtration of the sample was complete, the filter was washed 3 times with a volume of deionised water equal to the sample volume. Each filter was then returned into its original beaker and dried to constant weight in a Hot Box convection oven at 103 to 105°C (Gallenkamp, Leicester, UK). The beakers and filters were then cooled to room temperature in a desiccator and weighed to 4 decimal places. The total suspended solids were calculated as in equation 21.

$$SS\ [g\ l^{-1}] = (A - B) * 1000 / V \qquad \textit{Equation 21}$$

Where:

SS: suspended solids

A: weight of beaker, suspended solids and filter [g]

B: weight of beaker and filter [g]

V: sample volume [ml]

For determination of the volatile suspended solids the beakers and filters were then incinerated at 500±50°C for at least 1 hour. After incineration the beakers were cooled in a desiccator and weighed to 4 decimal points. The volatile suspended solids were calculated as in equation 22.

$$VSS\ [g\ l^{-1}] = SS - ((A - B) * 1000 / V) \qquad \textit{Equation 22}$$

Where:

VSS: volatile suspended solids

SS: suspended solids [g $l^{-1}$]

A: weight of beaker and filter with sample after incineration [g]

B: weight of beaker and filter [g]

V: sample volume [ml]

### 2.2.7 *Total organic carbon (TOC)*

The inorganic carbon and total organic carbon were determined with a DC-190 High Temperature TOC analyser from Rosemount Dohrmann (Santa Clara, US). For determination of total carbon 100 µl sample was manually injected into the analyser's combustion tube via an air-actuated injection port. The air flow through the platinum catalyst was set to 197 ml $min^{-1}$, the furnace was maintained at 680°C. For determination of inorganic carbon a 100 µl sample was manually injected via an air-actuated injection port into the IC reactor containing 20% phosphoric acid solution. The TOC analyser has according to the manufacturer an accuracy of +/- 0.4 mg C $l^{-1}$ and was calibrated weekly with 1000 mg C $l^{-1}$ potassium hydrogen phthalate ($C_8H_5KO_4$) standard for TC and 1000 mg C $l^{-1}$ $NaHCO_3$ standard for IC as recommended by the manufacturer. Reactor samples were diluted 1 in 10 before analysis. For TOC determination of the total sample flocs in the sample were broken down by repeated forcing of the sample through a syringe needle. For TOC determination of solubles only, the sample was filtered through a Whatman GF/C filter (pore size 1.2 µm) prior to analysis.

### 2.2.8 *Total starch*

The total starch was determined with a total starch assay kit from Megazyme Ltd. (Bray, Ireland) as in a method described by McCleary *et al.* (1997), with small adjustments for liquid samples. Megazyme Ltd. states that the method has an accuracy of ± 2%. The starch molecule is hydrolysed by α-amylase and amyloglucosidase. The resulting glucose units are determined colorimetrically. The method therefore determines the sum of starch, maltodextrin and glucose concentration and is used with the assumption that glucose and maltodextrin concentrations in the sample are negligible, which was tested and confirmed for glucose in experiment St1.

A 1 ml aliquot of undiluted sample (containing up to 10 g $l^{-1}$ starch) was mixed with 0.2 ml of 80% ethanol (90% v/v from Fisher Scientific, Loughborough, UK, diluted with deionised water) in a 12.5 cm screw cap test tube. In each batch 10mg maize starch standard (included in the assay kit, starch content 98% d.w.) suspended in 1ml deionised water was included for reference.

To each test tube 3 ml of thermostable α-amylase (100 U $ml^{-1}$, included in the kit) in MOPS buffer (50 mM, pH 7.0, made up from 99.5% MOPS sodium salt from Sigma, Gillingham, UK, pH adjusted with 1M HCl) was added, followed by 6 min. incubation in a boiling water bath. Before, during (after 3 minutes) and after incubation the tubes were stirred on a vortex stirrer. Then 4 ml sodium acetate buffer (200 mM at pH 4.5, made up from analytical grade glacial acetic acid from Fisher Scientific, Loughborough, UK, pH adjusted with 1M NaOH) and 0.1 ml amyloglucosidase in p-nitrophenyl β-maltoside (200 U $ml^{-1}$, pH4.5, included in the assay kit) were added to all test tubes. The tubes were stirred again and incubated in a water bath at 50°C for 30 minutes. After incubation the volume in all test tubes was made up to 10 ml before centrifuging for 10 minutes at 3000 rpm in a Centaurl bench top centrifuge (MSE Scientific Instruments, Crawley, UK) or a Sorvall Legend™ T centrifuge (Kendro Laboratory Products Plc, Bishop's Stortford, UK). After centrifuging duplicate 100 µl aliquots of the supernatant were transferred into 10 cm screw cap test tubes. In each batch duplicate standards of 100 µl glucose (included in the assay kit, 100 µg 0.1 $ml^{-1}$ in 0.2% benzoic acid) and 100 µl deionised water were included. Then 3.0 ml GOPOD (>12000 U $l^{-1}$ glucose oxidase and >650 U $l^{-1}$ peroxidase in reagent buffer, included in the kit) was added to all samples and standards, followed by incubation in a water bath at 50°C for 20 minutes. Absorbance was determined with a spectrophotometer (UV1 from Unicam, Cambridge, UK) at 510 nm 20 to 60 minutes after the GOPOD was added. If absorbance of the starch standard deviated no more than 10% from the glucose standard, starch concentrations of the samples were calculated from the two-point calibration of starch standard and blank. If the deviation exceeded 10% the analysis was repeated. Duplicate samples differed by less than 5%.

### *2.2.9 Total sugars*

Total sugar in experiments with sucrose and sugarbeet as substrate was determined with the phenol-sulphuric acid assay (Chaplin and Kennedy 1986) with minor alterations.

Prior to analysis duplicate samples were diluted to a hexose concentration of 20 to 200 mg $l^{-1}$ for maximum accuracy of the method. Samples containing particulate matter were stirred on a vortex stirrer to maximise the amount of sugar dissolved, and particulates then removed by centrifuging at 3000 rpm in a Centaur 1 bench top centrifuge (MSE Scientific Instruments, Crawley, UK) or a Sorvall Legend™ T centrifuge (Kendro

Laboratory Products Plc, Bishop's Stortford, UK). For calibration sucrose standards were made up by dissolving 1 g sucrose (analytical grade from Fisher Scientific, Loughborough, UK) in 1 litre of deionised water, followed by further dilution to 5 duplicate standards in the range of 0 to 200 mg $l^{-1}$. The $R^2$ value for the calibration curve always exceeded 0.990.

For analysis 400µl dilute sample or standard, 400 µl 5% w/v phenol (90% liquid phenol from Fluka, Buchs, Switzerland diluted in ultrapure water) and 2 ml sulphuric acid (98% analytical grade from Fisher Scientific, Loughborough, UK) were pipetted into a 10 cm screw-cap test tube. The tubes were left to cool in the fume cupboard for 10 min, then caps were screwed on, and the tubes inverted repeatedly. After a further 30 min the absorbances of standards and samples were determined with a spectrophotometer (UV1 from Unicam, Cambridge, UK). Sample concentrations were calculated from the 5 point calibration.

### *2.2.10 Sucrose*

The sucrose concentration in reactor contents (experiment Su5) and sugarbeet samples (section 2.7.2) was determined with an ENZYTEC™ sucrose/D-glucose/D-fructose enzymatic assay kit (Adgen Ltd, Ayr, Scotland), following the method provided with the kit (Schmidt 1961). Sucrose is hydrolysed by β-fructosidase to D-glucose and D-fructose. D-glucose is converted by hexokinase to glucose-6-phosphate, which is then oxidised by glucose-6-phosphate dehydrogenase to glucono-1,5-lactone 6-phosphate, a reaction which is coupled to reduction of added $NADP^+$. Therefore 1 mol of NADPH was formed per 1 mol sucrose. The NADPH was quantified colorimetrically.

Samples were diluted to give estimated sucrose concentrations of 80 to 800 mg $l^{-1}$ and centrifuged at 3000 rpm for 10 min to remove particles. 5 sucrose standards (analytical grade sucrose from Fisher Scientific UK) in the range 0 to 800 mg $l^{-1}$ were prepared per batch. All samples and standards were analysed in duplicates.

100 µl sample supernatant or standard and 200 µl citrate buffer (pH 4.6) containing 80 U $ml^{-1}$ β-fructosidase (provided with the assay kit in concentrate form) were pipetted into UV-grade disposable cuvettes, mixed and let to stand at 20 to 25 °C for 15 min.

Then 1.7 ml deionised water and 1ml triethanolamine buffer (pH 7.6) containing 2.6 mg $ml^{-1}$ NADP, 6.1 mg $ml^{-1}$ ATP and unquantified $MgSO_4$ (all provided as one powder with the assay kit) was added. The samples/standards were mixed and let to stand for 3 min, after which the absorbance was measured at 340 nm (UV1 from Unicam, Cambridge, UK) to quantify any natural NADPH.

Then 20 µl ammonium sulphate solution containing approximately 290 U $ml^{-1}$ hexokinase and 140 U $ml^{-1}$ glucose-6-phosphate dehydrogenase were added, the samples/standards were mixed and let to stand for 10 to 15 minutes, after which the absorbance at 340 nm was read again to quantify the sum of natural and produced NADPH.

The absorbance of natural NADPH was deducted from the final absorbance reading, then readings for duplicate samples were averaged. To determine the free glucose in the sample, the assay was also completed for each sample without addition of the β-fructosidase. To quantify sucrose only the absorbance of free glucose was deducted from the absorbance of glucose+sucrose. Sucrose concentrations were calculated from the 5-point calibration curve obtained from the sucrose standards. The $R^2$ value of the calibration curve exceeded 0.990 in all batches.

### *2.2.11 Cellulose and hemicellulose*

Cellulose and hemicellulose of the frozen grass used for experiments Gr1 to Gr3 were determined with the method of Allen *et al.* (1974), with minor alterations: half the recommended sample size was used due to restricted space in the centrifuge. Amounts of all reagents were also halved. For holocellulose determination grass with an estimated dry weight of 0.25 g was placed into duplicate 30 ml Pyrex centrifuge tubes. 15 ml deionised water, 0.15 g sodium chlorite (technical grade from BDH, Poole, UK) and 0.5 ml 10% (v/v) acetic acid (analytical grade from Fisher Scientific, Loughborough, UK) were added. Sample and reagents were mixed and then incubated in a water bath at 75°C for 4 hours. After 1, 2 and 3 hours further aliquots of sodium chlorite and acetic acid were added and any water lost due to evaporation was replaced. After incubation the tubes were cooled in ice water and then centrifuged at 4000 rpm for 15 min. The supernatant was decanted and the pellet resuspended in 15 ml deionised water. This was again centrifuged at 4000 rpm for 15 min. The washing procedure was then repeated once with deionised water, twice with acetone (analytical grade from

Fisher Scientific, Loughborough, UK) and once with diethyl ether (analytical grade from Fisher Scientific, Loughborough, UK). After the last wash the diethyl ether was allowed to evaporate over night and then the total and volatile solids of the pellet were determined as described in section 2.2.6, but the pellet was kept in the centrifuge tube throughout the process instead of transferring it into a beaker. The volatile solids of the pellet were defined as holocellulose.

For determination of the α-cellulose a duplicate sample of shredded frozen grass with an estimated dry weight of 0.5 g was placed into duplicate 30 ml Pyrex centrifuge tubes. 15 ml deionised water, 0.3 g sodium chlorite (technical grade from BDH, Poole, UK) and 1.0 ml 10% (v/v) acetic acid (analytical grade from Fisher Scientific, Loughborough, UK) were added. The samples were then incubated and washed as described for holocellulose determination. After the diethyl ether evaporated, 10 ml of 24% potassium hydroxide (analytical grade from BDH, Poole, UK) was added to the pellets. The tubes were then incubated in a water bath at 20°C for 2 hours, gently swirled at intervals. After incubation the tubes were again centrifuged at 4000 rpm for 15 min and the supernatant decanted. The pellet was washed as described for holocellulose. After the last wash the diethyl ether was evaporated over night, and the total and volatile solids of the remaining pellets were determined as for holocelullose. The volatile solids of the potassium hydroxide treated sample were defined as the α-cellulose fraction. The hemicellulose concentration was derived by subtracting the α-cellulose concentration from the holocellulose concentration.

### 2.2.12 Hydrogen yield

The hydrogen yield in mol hydrogen per mol hexose converted during continuous operation was calculated with equation 23.

$$H_2 yield = \frac{F_g \times \%H_2 \times Mr \times HRT \times 60 \min h^{-1}}{100\% \times (22.4l/mol) \times (C_{hi} - C_{ho}) \times V_r}$$

***Equation 23***

*with*

| | | |
|---|---|---|
| $F_g$ | *total gas flow rate [l min$^{-1}$]* |
| $\%H_2$ | *percentage hydrogen in the produced gas [%]* |
| $Mr$ | *molecular weight of hexose [g mol$^{-1}$]* |
| $HRT$ | *hydraulic retention time [h]* |
| $C_{hi}$ | *concentration of hexose entering reactor [g l$^{-1}$]* |
| $C_{ho}$ | *concentration of hexose leaving reactor [g l$^{-1}$]* |
| $V_r$ | *reactor volume [l]* |

Yields were calculated as daily average or average over a given time. For calculation of hydrogen yields (in mol hydrogen per mol hexose converted) in batch studies equation 23 could be simplified to equation 24.

$$H_2 yield = \frac{G \times \%H_2 \times Mr}{100\% \times (22.4l/mol) \times (S_i - S_o)}$$

***Equation 24***

*with*

| | | |
|---|---|---|
| $G$ | *total gas production [l]* |
| $\%H_2$ | *average of percentage hydrogen in the produced gas [%]* |
| $Mr$ | *molecular mass of hexose [g mol$^{-1}$]* |
| $S_i$ | *total hexose added [g]* |
| $S_o$ | *residual hexose [g]* |

## 2.3 *Feed*

The feed was made up in separate components: the main source of carbohydrate, a mineral nutrient solution and any additional organic substrates (where added) were kept in separate storage containers and pumped to the reactor through separate peristaltic pumps. Tap water for dilution was supplied directly from the mains through a self-refilling tank, and its flow rate was also controlled by a peristaltic pump. If more components were added than entry ports were available, the tubing for mineral nutrients, for dilution water and for additional organic substrates was joined immediately before the entry port.

### *2.3.1 Starch*

The starch used was a viscous, cold-swelling low-grade wheat starch co-product (DEFINOL BHW from Crespel & Deiters GmbH & Co. KG, Ippenbueren, Germany). Starch composition as provided by the suppliers is shown in Table 2-1. According to this data the wheat starch co-product has a higher starch content than the average of 63.9% given for wholemeal flour by the Flour Advisory Bureau or that of 70.1-73.4 % given for low grade wheat starch by Rank Hovis (section 1.7.3).

***Table 2-1. Properties of Definol BWH starch***

| | |
|---|---|
| Moisture content | Max. 10.0% |
| Protein content | Max. 5.0 % |
| starch content | Min. 75.0 % |
| ash content | Max. 0.8 % |
| pH value | Min. 4.0 |
| Particles > 315μm | Max. 0.2% |
| water absorption | Min 1: 6 |

The starch has various industrial uses, for example as core sand binders, thickener in drilling mud, binding agent in pellets and grill briquettes or binding agent and filler in the adhesive, paper and corrugated cardboard industry.

Analysis of triplicate samples gave a total solids content of 940 g $kg^{-1}$ and total starch content of 728 ± 31 g $kg^{-1}$, which is less than the minimum stated by the supplier.

The starch was blended with cold tap water to 80 g $l^{-1}$ in a domestic food processor (Moulinex, Paris, France) or industrial homogeniser (Kinematica AG, Littau-Lucerne, Switzerland) and stored at 4°C for up to 5 days. The dilution to 80 g $l^{-1}$ was found to allow maximum homogeneity during storage. Unless stated otherwise, the starch was diluted to 10 g $l^{-1}$ at the point of entry to the reactor.

### *2.3.2 Sugarbeet and refined sugar*

For experiments with refined sugar food-grade granulated cane sugar from Tate & Lyle (London, UK) or SilverSpoon granulated beet sugar (British Sugar, Peterborough, UK) was used. The sugar was dissolved in hot tap water to 200 g $l^{-1}$, stored at 4°C for up to 7 days and diluted to 10 g $l^{-1}$ at the point of entry to the reactor.

Whole sugarbeets were supplied by ADAS (Ely, UK). A first delivery of 5 kg sugarbeet was received in December 2002. This was used for experiments Su1 and Su2 only. In all following experiments sugarbeet from a second and third delivery received in January and February 2003 was used. The sugarbeet was washed, peeled and frozen within three days of arrival. For batch studies the required amount of substrate was defrosted in the fridge during the night before start-up, chopped into 1-3 cm pieces and pulped with equal amounts of tap water (1:1 w/w) in a domestic juicer (Moulinex, Paris, France). This pulp, consisting of whole pulped sugarbeet and water (1:1 w/w) will be referred to as "pulped sugarbeet", which was used for batch start-ups in experiments Su1 to Su7. For continuous operation batches of 1.5 to 2 kg of sugarbeet were defrosted and pulped as for batch start-up. However, since the fibre in the pulp blocked Marprene tubing in peristaltic pumps (Watson Marlow, Falmouth, UK) as well as the injection check valve in a microprocessor dosing pump (LMI, Winsford, UK), it was then extracted by manually squeezing it through a 185 μm mesh polyester fabric (Sericol Industrial Fabrics, Kent, UK). The same amount of water as added initially for pulping of the beet was then mixed with the remaining pulp, the resulting slush left to stand for 30 min, and then again squeezed through the fabric. This was repeated once more. All of the water extract was then filtered through a 60 μm mesh polyester fabric (Sericol Industrial Fabrics, Kent, UK), and the remaining fine pulp added to the rest of the extracted pulp. Water extract and extracted pulp were then frozen separately in aliquots. The water

extract was fed continuously with a peristaltic pump (Watson Marlow, Falmouth, UK), whilst the extracted pulp was injected twice daily by syringe through the liquid sampling port.

The sucrose content of water extract and extracted pulp was determined for each preparation batch, and flowrates of water and water extract were adjusted to achieve a sucrose feed concentration of 10 g $l^{-1}$ at the point of entry to the reactor. Extracted pulp was injected in the same ratio in which it was originally separated from the extract. For this the appropriate amount of pulp (approximately 35 g) was diluted to 100 ml with tap water. Addition of pulp reduced the HRT from 15 to 14.2 hours.

### *2.3.3 Grass*

Approximately 10 kg of rye grass of the variety AberDart was obtained from the Institute of Grassland and Environmental Research (IGER) in Aberystwyth (Wales, UK) in summer 2002. The grass was harvested on 29/30 May 2002 and stored at -20°C. The variety AberDart was bred and developed at IGER in Aberystwyth and was the first high sugar perennial ryegrass to become commercially available.

The release of total sugars from several types of pre-treatment was investigated. For all pre-treatments the frozen grass was first shredded to < 10 cm long pieces with an AXT 1600 HP garden material shredder from Bosch (purchased in B&Q, Pontypridd, UK) and re-frozen. Physical, chemical and enzymatic pretreatments as well as their combination were tested on the shredded grass and are described in the following sections. The range of pre-treatments tested and the carbohydrate quantity extracted are summarised in Table 5-1 (section 5.2).

#### 2.3.3.1 Physical pre-treatment (Methods No. 1 to 3 in Table 5-1)

After failed attempts to physically disrupt the grass with a domestic food processor and a domestic juicer (both from Moulinex, Paris, France), in both of which the grass wrapped itself around the blades, two pieces of equipment were purchased for this purpose: a domestic food waste disposer / in sink erator from Emerson Electric Co. (Great Dunmow, UK), and a domestic multipurpose juicer from Matstone (purchased from Outspan Fresh, Maidenhead, UK). Both instruments could only process a few

grams of grass at a time, thus they are not suitable for processing of higher volumes of grass, such as are required for continuous operation. Also, for processing in both instruments the grass had to be suspended in water beforehand, and further addition of water during processing was necessary to prevent blockage of either instrument. 20 to 60 g wet grass (approximately 10 to 30 g dry weight) mixed with 1 litre tap water were therefore gradually fed through each instrument. As the food waste disposer was less efficient in disrupting the grass structure (as concluded from visual examination), grass plus extract were put twice through the food waste disposer, but only once through the juicer. After physical pre-treatment the disrupted grass was separated from the extract by filtering through a 185 μm mesh polyester fabric (Sericol Industrial Fabrics, Kent, UK).

#### 2.3.3.2 Chemical pre-treatment (Methods No. 4 and 5 in Table 5-1)

An alkaline peroxide treatment based on the method by Gould (1983) for pre-treatment of corn stover and wheat straw and its optimisation for steam-exploded softwood by Yang *et al.* (2002) was tested for grass pre-treatment. As discussed in the introduction alkaline peroxide pre-treatment was expected to extract lignin and hemicellulose.

For this 4.3 g shredded wet grass (to give approximately 2 g dry weight) was added to 100 ml of 1% $H_2O_2$ (100 vol laboratory grade $H_2O_2$ from Fisher Scientific, Loughborough, UK, diluted in tap water) in duplicate 250 ml conical flasks and adjusted to pH 11.5 with 1M NaOH. The flasks were then incubated at 30°C and 120 rpm in an orbital incubator from SANYO Gallenkamp (Leicester, UK) for 43 hours, or at 80°C in a water bath with regular manual stirring for 45 minutes. After incubation the residual grass was separated from the extract by pouring the sample through a 1.18 mm mesh soil sieve.

2.3.3.3 Enzymatic pre-treatment (Methods No. 6 to 8 in Table 5-1)

A range of enzymes used for digestion of lignocellulose materials was supplied by Biocatalysts Ltd. (Treforest, UK). The effect of a combination of the following enzymes on grass was tested in concentrations as recommended by the supplier:

- D740L: ferulic acid esterase from *Humicola sp.*, opt. pH 4-6, 40-65°C
  36 U $ml^{-1}$, added as 0.2 ml per kg dry grass
- C013L: cellulase from *Trichoderma sp.*, opt. pH 3.5-6.0, 50-70°C
  1500 U $ml^{-1}$, liquid added as 5% v/w dry grass
- D453P: xylanase from *Aspergillus niger*, opt. pH 4.0-5.5, 40-60°C
  5000 U $g^{-1}$, powder added as 600 ppm dry grass
- D333P: pentosanase from *Trichoderma reesei,* opt. pH 4.0-5.5, 30-55°C
  11000 U $g^{-1}$, powder added as 1% w/w dry grass

100 ml citric acid buffer at pH 5.2 (13.6 ml 0.05M citric acid made up to 100 ml with 0.05M trisodium citrate) was added to 5 g wet grass in duplicate 250 ml conical flasks. A combination of above enzymes was added, and the flasks were incubated in the orbital incubator from SANYO Gallenkamp (Leicester, UK) at 50 °C for 24 hours. After incubation the residual grass was separated from the extract by pouring the sample through a 1.18 mm mesh soil sieve.

2.3.3.4 Combinations of treatment (Methods No. 9 to 15 in Table 5-1)

It was thought that physical pre-treatment would improve the efficiency of chemical and enzyme treatment by breaking up cell wall structures and thus giving chemicals and enzymes better access sites to degradable bonds. Equally, it was thought that alkaline peroxide pre-treatment may improve the access for enzymes by removing the lignin possibly coating the cellulose network. Therefore the three extraction methods were tested in series in several combinations. For this the following alterations and additions to the extraction process were made:

- The initial dry matter concentration was approximately 30 g $l^{-1}$. Although both Gould (1983) and Yang *et al.* (2002) optimised their methods for 20 g $l^{-1}$ dry weight, Gould (1983) actually states that the method was found to be equally efficient for up to 40 g $l^{-1}$dry weight. 30 g $l^{-1}$ dry weight was found to give the maximum volume of grass that was easily mixable in the liquid.

- Where physical treatment was followed by alkaline peroxide treatment, the extract and residual grass were not separated at the end of the physical treatment, but the pH of the grass and extract together was adjusted to 11.5 and then 100 vol. $H_2O_2$ was added to give a $H_2O_2$ to substrate ratio of at least 0.5 (w/w).
- Where alkaline peroxide treatment was followed by enzymatic treatment, the extract and residual grass were not separated at the end of the chemical treatment. The residual $H_2O_2$ was removed before enzymes were added. For this, the sample pH was checked and if necessary adjusted to ~7 with 1M HCl, before approximately 1 ml of Catalase 495L (from Biocatalysts Ltd, Trefforest, UK) was added to remove any remaining $H_2O_2$. The catalase specification on certificate of analysis states that on 05/12/03 the actual activity was 26 334 U/g, determined from one unit of enzymes decomposing 1μmol $H_2O_2$ $min^{-1}$ at pH 7.0 and 25°C (the catalase was used between 20/02/04 and 25/03/04). Once oxygen production ceased, the pH of the sample was adjusted to 5.5 with 1 M HCl (instead of suspension of the grass in citric acid buffer as described in section 2.3.3.3) before the lignocellulose degrading enzymes were added.

The final method for extraction of the grass used in the experiments with grass extract described in this thesis (Gr 1, Gr2 and Gr 3) was as follows:

1. 120 g shredded grass and 2 l tap water was put through the domestic multipurpose juice extractor from Matstone. Liquid and solid were treated together in the following steps
2. 1 M NaOH was added to bring pH to 11.5
3. 104 ml 30% $H_2O_2$ was added to give a $H_2O_2$ concentration of approximately 1.5%.
4. Once foaming started to decrease (after 10-15 min), the extraction batches were incubated in a waterbath at 80°C for 45 min, timed from the point at which the liquid temperature exceeded 70°C.
5. After cooling to <55°C, the pH was checked and if necessary adjusted to ~7 with 1M HCl.
6. Approximately 1 ml of Catalase 495L was added to remove any remaining $H_2O_2$.
7. Once oxygen production ceased, the pH was adjusted to 5.5 with 1 M HCl, 0.1 ml D740L, 3.0 ml C013L and 0.036 g D453P were added and the batches incubated for 26 (Exp. Gr3) or 48 h (Exp. Gr1 and Gr2) at 50°C

The solids were removed by filtering through the 185μm polyester mesh, and the grass extract (=filtrate) frozen for up to 1 week before use.

### *2.4 Nutrients*

Mineral nutrients were added to the carbohydrate substrate in all experiments. Two different nutrient solutions were used. The composition of both solutions is given in Table 2-2. Nutrient solution A was used by Mizuno, *et al.* (2000a), and was used here for experiment St2 only. Nutrient solution B, which was used in all other experiments, was as used by Fang and Liu (2002) except for $NH_4Cl$, which was maintained at the concentration used in solution A rather than at 500 mg $l^{-1}$, as given by Fang and Liu (2002).

***Table 2-2. Composition of nutrient solutions [mg $l^{-1}$] at point of entry to reactor***

| Salt | Solution A [mg $l^{-1}$] | Solution B [mg $l^{-1}$] |
|---|---|---|
| $NH_4Cl$ | 2600 | 2600 |
| $K_2HPO_4$ | 250 | 250 |
| $KH_2PO_4$ | - | 250 |
| $MgCl_2 \cdot 6H_2O$ | 125 | 320 |
| $FeSO_4 \cdot 7H_2O$ | 5 | 86 |
| $CoCl_2 \cdot 6H_2O$ | 2.5 | 15 |
| $MnCl_2 \cdot 4H_2O$ | 2.5 | 30 |
| KI | 2.5 | - |
| $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ | - | 14 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.5 | - |
| $Na_2B_4O_7 \cdot 10H_2O$ | - | 12 |
| $H_3BO_3$ | 0.5 | - |
| $NiCl_2 \cdot 6H_2O$ | 0.5 | 49 |
| $ZnCl_2$ | 0.5 | 23 |
| $CuCl_2 \cdot 2H_2O$ | - | 10 |
| $CaCl_2 \cdot 2H_2O$ | - | 66 |

The solutions were made as 13 times concentrated and acidified to pH<2. Approximately 1 ml polymethylsiloxane based AP Antifoam (Aquatreat, Llanelli, Wales) was added per 5 l concentrated nutrient solution.

As listed in Table 2-3 (experiments with starch), peptone and glucose were added in some experiments with starch. Both were added at the final concentration of 1 g $l^{-1}$. The

peptone was either granulated dry meat extract from Merck (Darmstadt, Germany) or peptone from Fisher Bioreagents (Loughborough, UK), made up fresh every 3 days as 20 times concentrated in tap water and stored at 4°C. The glucose was anhydrous D-glucose for general lab use from Fisher Scientific (Loughborough, UK), made up weekly as 20 times concentrated in tap water and stored at 4°C.

### *2.5 Inoculum*

Anaerobic digester sludge from Cog Moors sewage treatment works (Cardiff, UK) was used as inoculum for all experiments. For experiments on starch and sugarbeet with exception of experiments St8 and St9 the sludge was collected from a continuously fed stabilisation tank into which the effluent of the anaerobic digester was fed at 7 d HRT. At the time when experiments with sugarbeet were completed, the operation of this stabilisation tank was changed from continuous mode at 7 d HRT to batch mode with up to 14 days holding time. As it was thought that this might affect the bacterial population in the tank, for experiments on grass extract and experiments St8 and St9 the sludge was collected from the transfer pipe between anaerobic digester and stabilisation tank. After collection the sludge was sieved through a 1.18 mm mesh soil sieve and stored in a sealed container at room temperature for up to 7 months.

### *2.6 Start-up*

For batch start-up sufficient sludge was measured out to give 3 g $l^{-1}$ TS in the reactor. For heat treatment to >90°C the volume of sludge to give 3 g $l^{-1}$ TS was heated in a conical flask in a domestic pressure cooker filled with water to the sludge level and with a loosely placed lid to let the steam escape. Once the sludge was heated to >90°C (tested with a digital thermometer inserted into the sludge), it was kept in the boiling water for a further 15 minutes. For heat treatment to 110°C the sludge was heated in the same pressure cooker but with the lid securely locked. Instead of the conical flask the sludge was contained in a 500 ml Pyrex media bottle. Once steam started forming, a 41.99g weight was fitted to the lid. From the point the safety valve shut, the cooker was heated for another 10 minutes.

The sludge (after cooling to <50°C if heat-treated) was mixed with the required volumes of substrate and nutrients and the pH adjusted to below 6 with hydrochloric acid. The

culture was then filled into the already temperature controlled reactor and made up with tap water to the required total volume (2.3 or 9.5 l). In experiments with nitrogen sparging, sparging was started before the reactor was filled, to prevent reactor contents penetrating and blocking the sparger pores. The reactor was sealed and the stirrer and pH control fitted and activated as quickly as possible. If required, AP antifoam (Aquatreat, Llanelli, Wales) diluted 1:1 with water was added through a port in the reactor lid. The point at which the culture was sealed and stirred was taken as time zero and datalogging with LabVIEW$^{TM}$ was started. If the operational mode was to be changed to continuous, the pumps supplying feed, nutrients and water continuously were switched on manually during working hours once measurable gas production had started.

## *2.7 Experiments*

### *2.7.1 Hydrogen production from a particulate wheat starch*

23 experiments were carried out using the wheat starch co-product as principle source of carbohydrate. Of these, 1 batch and 8 continuous experiments are described here. In the other 14 experiments technical problems made data analysis difficult. Of the 9 experiments described here, 1 batch and 5 continuous experiments were operated in a self generated gas atmosphere, and 3 continuous experiments were continuously sparged with nitrogen.

#### 2.7.1.1 Experiments St1 to St6 with self generated gas atmosphere

Table 2-3 summarises operating conditions and duration of the 6 experiments described here. For all experiments in this section sludge was heat-treated to >90°C for 15 minutes. Experiments were either operated at pH 4.5 and 35°C without added glucose and peptone, or at pH 5.2 and 30°C with continuous addition of 1 g $l^{-1}$ peptone and 1 g $l^{-1}$ glucose. Only in experiment St2 nutrient solution A was used, nutrient solution B in all others. The hydraulic retention time in the 4 continuous experiments was varied from 18 to 9 hours. "Batch start-up duration" in Tables 2-3 to 2-6 is the number of hours for which the reactor was operated in batch mode. This period depended on the lag phase to gas production, as continuous operation was only started once measurable gas production had started, and the time of day (continuous feeding was started manually between 8 a.m. and 5 p.m.).

***Table 2-3. Operating parameters, nutrients and duration of experiments on starch with self generated gas atmosphere***

| Exp. No. | pH | Temp. [°C] | HRT [h] | Nutrient Solution | Reactor | Batch start-up Duration [h] | Contin. feeding Duration [d] | Storage duration of inoculum [d] |
|---|---|---|---|---|---|---|---|---|
| Batch start-up | | | | | | | | |
| St1 | 4.5 | 35 | - | B | B | 55 | - | 20 |
| Continuous operation at pH 4.5, starch only | | | | | | | | |
| St2 | 4.5 | 35 | 18 | A | A | 20 | 14 | 34 |
| St3 | 4.5 | 35 | 18 | B | B | 40 | 9 | 32 |
| Continuous operation at pH 5.2, starch, glucose and peptone | | | | | | | | |
| St4 | 5.2 | 30 | 18 | B | B | 40 | 9 | 74 |
| St5 | 5.2 | 30 | 18 | B | B | 24 | 12 | 6 |
| | | | 12 | | | | 7 | |
| | | | 9 | | | | 3 | |
| St6 | 5.2 | 30 | 18 | B | A | 40 | 15 | 6 |
| | | | 12 | | | | 7 | |

2.7.1.2 Experiments St7 to St9 with nitrogen sparging

All 3 sparging experiments described here were performed in reactor A at pH 5.2, 15 h HRT and 32°C, with nutrient solution B and added peptone. Heat-treatment of inoculum, substrate concentration and glucose addition were changed as shown in Table 2-4.

***Table 2-4 Sludge pre-treatment, substrate and duration of experiments on starch with nitrogen sparging (pH5.2, 15 h HRT, 32°C)***

| Exp. No. | Heat treatment | Substrate conc. [g $l^{-1}$] | Batch start-up Duration [h] | Contin. feeding Duration [d] | Storage duration of inoculum [d] |
|---|---|---|---|---|---|
| St7 | >90°C | 10 (+G)* | 43 | 18 | 147 |
| St8 | 110°C | 20 | 23 | 8 | 55 |
| St9 | 110°C | 20 | 24 | 9 | 15 |

*G: 1 g $l^{-1}$ glucose

On days 6 and 9 of experiment St9 L-cysteine hydrochloride hydrate (Fisher Scientific, Loughborough, UK) dissolved in 10 ml tap water was injected into the reactor through the feed port to give a concentration of 0.1% cysteine in the reactor liquid.

For sparging in these experiments a shortened sinter stick was used as gas disperser. For experiment St7 this was a sinterstick with unknown pore size and supplier. The sparging rate (estimated with Cole Parmer flow meter, see section 2.1.3.1) varied from 50 to 65

ml $min^{-1}$ and tended to decrease naturally during the experiment, probably due to gradual growth of microorganisms on the sinter sticks, decreasing the porosity. A maximum sparging rate of 65 ml $min^{-1}$ (roughly 10 times the gas production rate) gave a hydrogen content in the exiting gas of around 5%, which is near the detection limit of the GC used for analysis. For experiments St8 and St9 the sinterstick used in experiment St7 was replaced with a shortened sinter stick from Bibby Sterilyn (purchased from Fisher Scientific, Loughborough, UK) with a defined pore size of 10 to 16μm. It created smaller bubbles than the sinterstick used for experiment St7, but the finer pore size also had the undesired side effect that a gas pressure of up to 40 kPa (measured with a compensated gauge pressure sensor from Honeywell, purchased from Farnell, Leeds, UK) had to be applied to achieve a gas flow rate of ~60 ml $min^{-1}$ through the sinter stick. Due to the increased gas pressure the ingoing gas flow data for the sparging rate was not used for any calculations and is not shown in figures.

Hydrogen yields for all sparging experiments were calculated with equation 25, for which sparging rate is not required. Hydrogen and carbon dioxide data for experiments with sparging are displayed in the form of production rates [ml $min^{-1}$ $l^{-1}$], which were calculated from the total gas flow out [ml $min^{-1}$ $l^{-1}$] and its hydrogen/carbon dioxide content [%]. For online measurements this calculation was performed for each logged data point. For offline hydrogen this calculation was performed for each hydrogen content data point. Since gas sampling for hydrogen determination affected the total gas flow at that point in time, the average gas flow during the 20 minutes preceding the gas sampling was used for calculation of the hydrogen production rate.

### 2.7.2 *Hydrogen production from sugarbeet and refined sugar: Experiments Su1 to Su10*

The experiments described in this section examined the feasibility of continuous hydrogen production from sugarbeet and refined sugar in batch and continuous operation with and without nitrogen sparging. Redox potential, fermentation end products, liquid phase mass balance and hydrogen yields during continuous operation were determined.

10 experiments were carried out, using pulped whole sugarbeet, sugarbeet extract with or without the extracted pulp or refined sugar as substrate. Of these, five experiments were batch studies, whilst the other five batch start-ups were converted to continuous operation. Details on inoculum storage duration, substrate type and experiment duration are given in Table 2-5. Operating pH and temperature were 5.2 and 32°C for all experiments, the retention time during continuous operation was 12 or 15 hours. Anaerobic digester sludge was used as inoculum for all experiments, but was heat-treated at >90°C for 15 min for experiments Su1 to Su7 only. Nutrient solution B was added to the substrate in all experiments.

For batch start-up with pulped whole sugarbeet (Su1 to Su7) 100 g sugarbeet pulped with 100 ml water was used as substrate. In experiment Su4 the pulped beet was heated in a water bath and maintained at >90°C for 15 min. In experiment Su5 it was autoclaved at 121°C and 1.05 bar for 22 min with a Prestige Medical Omega autoclave (Blackburn, UK). To determine whether autoclaving affected the sucrose concentration of the substrate, the sucrose concentration of one pulped piece of sugarbeet (1:4 w/w with water) was analysed in duplicate before and after autoclaving, using the enzymatic method described in section 2.2.10. It was found to contain 18.1% and 18.6% sugar before, and 19.1% and 19.9% sugar after autoclaving, which suggests that autoclaving might increase the accessibility of some of the sugar in the pulped sugarbeet. In experiments Su9 and Su10, the feed was changed from refined sugar to sugarbeet water extract with or without extracted pulp during periods of operation as shown in Table 2-5.

***Table 2-5. Operating parameters and substrate for experiments with sugarbeet and refined sugar***

| Exp | Batch (start-up) | | | | Continuous operation | | | |
|---|---|---|---|---|---|---|---|---|
| | Sludge Storage time [d] | Duration [h] | Sparging | Substrate | Duration [days from start-up] | HRT [h] | Sparging | Substrate |
| Su1 | 26 | 65 | No | Pulped whole sugarbeet | - | | | |
| Su2 | 43 | 48 | No | Pulped whole sugarbeet | - | | | |
| Su3 | 58 | 47 | No | Pulped whole sugarbeet | - | | | |
| Su4 | 65 | 46 | No | Pulped whole sugarbeet, Heat-treated | - | | | |
| Su5 | 72 | 49 | No | Pulped whole sugarbeet, autoclaved | - | | | |
| Su6 | 84 | 24 | No | Pulped whole sugarbeet | 1 to 5 | 12 | No | Refined sugar |
| Su7 | 100 | 27 | No | Pulped whole sugarbeet | 1 to 8 | 12 | No | Refined sugar |
| Su8 | 119 | 74 | No | Refined sugar | 3 to 10 | 15 | No | Refined sugar |
| Su9 | 21 | 24 | Yes | Refined sugar | 1 to 17 | 15 | Yes | Refined sugar |
| | | | | | 17 to 34<br>38 to 41 | 15 | No | refined sugar |
| | | | | | 34 to 38<br>41 to 45 | 15 | No | Sugarbeet extract & extracted pulp |
| Su10 | 5 | 23 | Yes | Refined sugar | 1 to 11<br>18 to 25 | 15 | Yes | Refined sugar |
| | | | | | 11 to 18<br>25 to 32 | 15 | Yes | Sugarbeet extract |

Nitrogen sparging was only applied in experiments Su9 and Su10. In experiment Su9 sparging was stopped after 17 days. For both experiments the same sinter stick as in

experiment St8 and St9 was used for gas dispersion. The sparging rate varied from 40 to 70 ml $min^{-1}$ throughout the experiments with a tendency to decrease naturally during experiments Su9 and Su10 as observed in experiment St7 to St9.

### *2.7.3 Hydrogen production from grass: Experiments Gr1 to Gr3*

Prior to experiments with grass extract investigations were undertaken to determine if grass shredded in the garden material shredder (see section 2.3.3) directly suspended in water at 10 g dry matter per litre reactor volume could be stirred as described in section 2.1.1. The grass wrapped itself around the stirrer and brought it repeatedly to a halt within 2 hours operation. The stirrer was then removed, and the grass and water were mixed by recycling of the head-space gas at a rate of 100 ml $min^{-1}$ $l^{-1}$ reactor volume by a peristaltic pump (Watson Marlow, Falmouth, UK) from the gas sampling tube to the sinterstick at the reactor base. This resulted in gas bubbles forming around the grass blades, causing the grass to float, wrap itself around the pH probe, inhibiting pH control and blocking the gas tube leading from the reactor head-space to the instruments. It was concluded that grass could not be used directly as substrate in the existing reactors. Therefore efforts were concentrated on use of grass extracts.

5 experiments with grass extract (see section 2.3.3.4 for extraction method) were completed, of which 3 will be discussed in this thesis. The omitted experiments suffered from technical problems, which made data interpretation difficult. Because of the labour intensive pre-treatment required to produce an extract which could be handled at lab scale and contained cell wall components as well as non-structural carbohydrate, hydrogen production from grass was demonstrated in batch mode only. The inoculum for all experiments was anaerobic digester sludge, heat-treated to 110°C for 15 min. The operating pH was 5.2 and the temperature 32°C, and only mineral nutrient solution B and antifoam were added to the extract. All experiments were conducted in reactor A and had all online instruments including hydrogen sensor fitted. For sparging the sinter stick from Bibby Sterilyn with defined pore size of 10 to 16µm was used. The sparging rate varied from a minimum of 45 to a maximum of 70 ml $min^{-1}$ $l^{-1}$ during the experiments. The exiting gas rate was determined with the ADM2000 flow meter from Agilent (Placerville, USA).

A summary of the 3 experiments described here is given in Table 2-6.

***Table 2-6. Batch experiments with grass extract ( pH 5.2 and 32°C)***

| Exp. No. | Duration | Sparged | Total sugar in grass extract [g $l^{-1}$] | Initial total sugar in reactor [g $l^{-1}$] |
|---|---|---|---|---|
| Gr1 | 70 h | No | 12.6 | 10.2 |
| Gr2 | 72 h | Yes | 10.5 | 14.0 |
| Gr3 | 95 h | Yes | 7.6 | 6.2 |

Since hydrogen production ceased by hour 40 in all 3 experiments, results are shown for hours 0 to 45.

For experiment Gr3 3 samples were sent to the Institute of Grassland and Environmental Research (Aberystwyth, Wales) for analysis of fructan, neutral polymers, sucrose, glucose, galactose and fructose by HPLC with refractive index detector after purification on cation and anion exchange resins, following the method of Cairns *et al.* (2002). The following samples were sent:

a) the grass extract used as substrate for this experiment
b) a reactor sample taken at hour 0
c) a reactor sample taken at hour 42.

# 3 Hydrogen production from a particulate wheat starch

The methodology for the experiments described in this section is given in section 2.7.1. Heat-treated anaerobic digester sludge was used as inoculum, since this has been shown a suitable inoculum for continuous hydrogen production from a soluble starch substrate (Lay 2000). Experiments St1, St3, St4, St6 and St7 were described in Hussy *et al.* (2003). Experiments St8 and St9 were described in Hussy *et al.* (2004). Publications are enclosed in the Appendix.

The objective of experiments on starch was initially to optimise continuous hydrogen production. Based on previous experience with pure glucose (Mizuno *et al.* 2000a; Dinsdale 2001) it was expected that stable continuous hydrogen production would be easily achieved and the research could focus on maximising hydrogen yields. However, as will be described in this section, continuous hydrogen production on particulate wheat starch was often highly variable and prone to failure. Therefore, whilst stable continuous hydrogen production from the particulate wheat starch is demonstrated in experiment St7, analysis and discussion of experiments St1 to St6 focuses also on the limitations to and interferences with stable continuous hydrogen production and on possible ways to decrease or remove these limitations.

## *3.1 Experiments with self generated gas atmosphere*

### *3.1.1 Batch start-up*

The dynamics of batch start-up were investigated through intensive monitoring of one complete batch study (Experiment St1; Figures 3-1a and 3-1b).

Figure 3-1a shows that there was a 9 hour lag phase to the beginning of detectable gas production. During the first part of gas production (hours 9 to 27) the hydrogen content of the produced gas (56.8 ± 1.8 %; n=11) exceeded the carbon dioxide content. During the period of highest gas production (hours 29 to 50) similar amounts of hydrogen (48.7 ± 1.4%; n=8) and carbon dioxide (50.4 ± 1.3%) were produced, accounting on average for 99% of the exiting gas. This shows that no/negligible amounts of methane were produced. A total of 1.4 mol hydrogen per mol hexose converted was produced during this batch experiment, with a total gas production of 2.6 litres per litre reactor volume.

From average hourly gas production rates and the offline hydrogen data a total hydrogen production of 12.8 litres (reactor volume: 9.5 l) can be estimated.

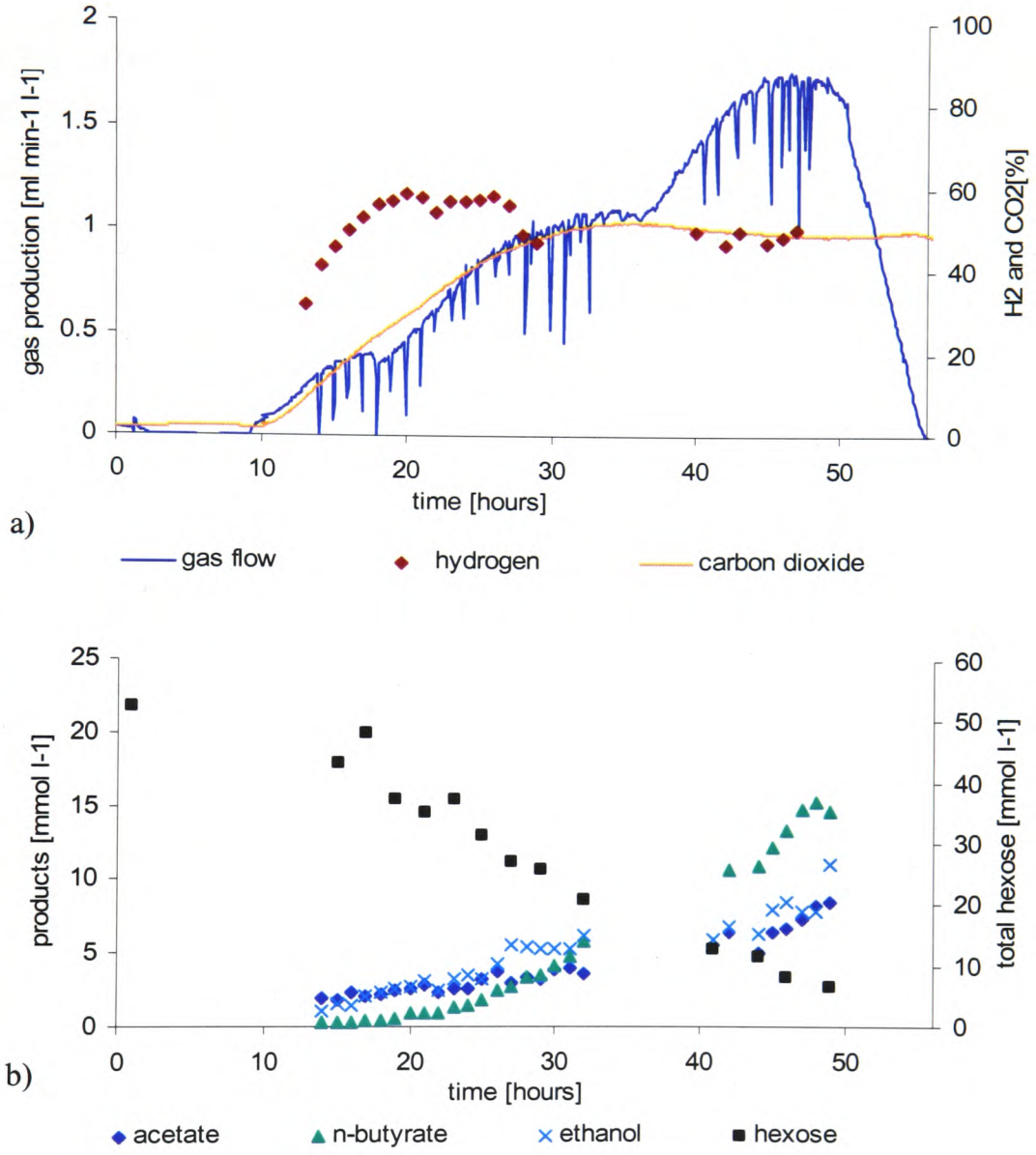



***Figure 3-1. Experiment St1. Batch study at pH 4.5 and 35°C. a) gas production [ml $min^{-1}$ $l^{-1}$], hydrogen and carbon dioxide content [%] b) products and residual total hexose [mmol $l^{-1}$]***

During the first part of the batch experiment up to hour 32 ethanol and acetate concentrations exceeded the butyrate concentration (Figure 3-1b). However, during the peak of gas production the butyrate concentration exceeded acetate and ethanol concentrations. At hour 48, by the end of gas production, butyrate was the main determined non-gaseous product with a peak value of 15.3 mmol $l^{-1}$ (Figure 3-1b), whilst a maximum of 8.5 mmol $l^{-1}$ acetate and 11.1 mmol $l^{-1}$ ethanol was produced. No

propionate, acetone or butanol was detected. Starch was converted at a steady rate throughout the batch experiment, and starch concentration had decreased to less than 6.5 mmol $l^{-1}$ by hour 49, when gas production started to decrease, giving a substrate conversion of at least 88%. Free glucose levels were never above 2.2 mmol $l^{-1}$.

A simple carbon balance (in mmol C $l^{-1}$) of converted substrate and determined products (Table 3-1) shows that only 54% of carbon converted (275 mmol C $l^{-1}$) was recovered in the determined products (148 mmol C $l^{-1}$). Some of this difference may be caused by an underestimate of the produced carbon dioxide due to the reactor headspace, but since the reactor headspace holds approximately 1.5 litre gas compared to a total of 21 litre produced gas, this can only explain a small part of the unaccounted carbon.

***Table 3-1. Carbon balance for hours 0 to 49 of experiment St1.***

| | hour 0 [mmol $l^{-1}$] | hour 49 [mmol $l^{-1}$] | consumption/production hours 0 to 49 [mmol $l^{-1}$] | Carbon balance [mmol C $l^{-1}$] |
|---|---|---|---|---|
| Hexose | 52.3 | 6.49 | 45.8 | 274.9 |
| Butyrate | 0 | 14.7 | -14.7 | -58.8 |
| Acetate | 0 | 8.5 | -8.5 | -17 |
| Ethanol | 0 | 11.1 | -11.1 | -22.2 |
| Propionate | 0 | 0 | 0 | 0 |
| Acetone | 0 | 0 | 0 | 0 |
| Butanol | 0 | 0 | 0 | 0 |
| Carbon dioxide | 0 | 49.6[1)] | -49.6 | -49.6 |
| Σ C in products | | | | 147.6 |
| C consumed accounted for in products | | | | 53.7 % |

1) total of $CO_2$ produced hours 0 to 49

Therefore significant amounts of un-assayed other products (e.g. lactate) must have been formed. In absence of methanogenesis formation of ethanol and these unidentified products are most likely the cause for the difference between the achieved hydrogen yield of 1.4 mol per mol hexose converted and the theoretical hydrogen yield of 2 to 4 mol per mol hexose expected in association with butyrate/acetate production (equations 1 and 2).

### *3.1.2 Continuous operation at pH 4.5 and 35°C*

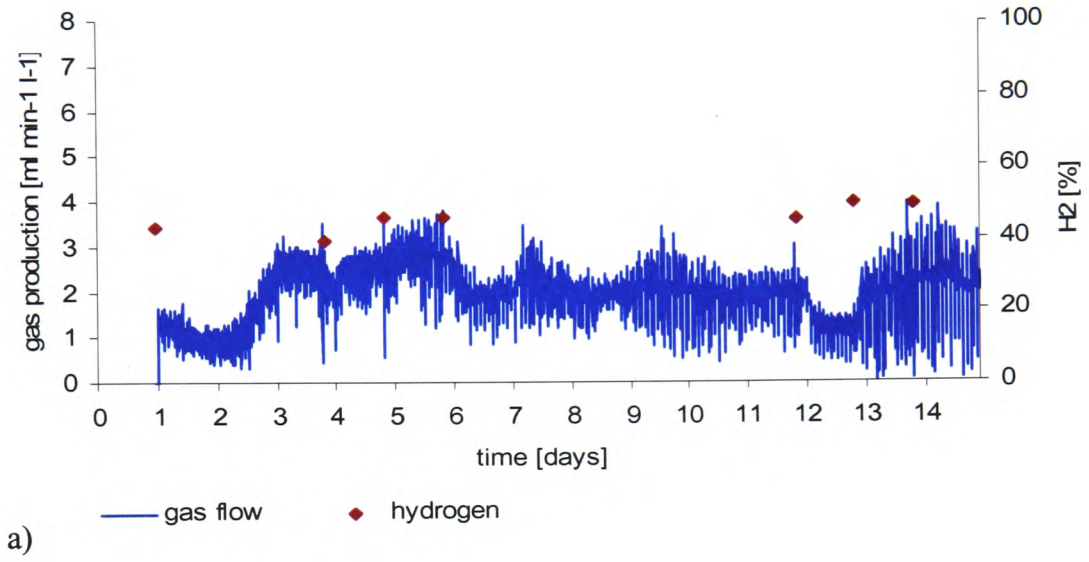
The first experiments which were converted to continuous operation, but are not included here because of the technical problems encountered, were controlled at pH 6, since this proved to be a suitable pH for continuous hydrogen production from glucose in this laboratory (Mizuno *et al.* 2000a). However, on starch, problems with propionate production as well as methanogenesis were observed at pH 6.0 (data not shown). For the 2 experiments described in this section, the pH was decreased to 4.5, to inhibit both propionate production (Ren *et al.* 1997) and growth of methanogens (Sahm 1984). The operating temperature was maintained at 35°C as in Mizuno *et al.* (2000a) since there are no indications in the literature that the optimum temperature is substrate dependent. Since Lay (2000) reported a HRT of 17h as optimum for continuous hydrogen production from a soluble starch, 18h HRT was chosen for the particulate substrate used here.

For experiment St2 nutrient solution A was used, for experiment St3 solution B (Table 2-3). Nutrient solution B was introduced because it was noticed that solution A contained low concentrations of several nutrients, particularly iron and also sulphur (Table 2-2) in comparison to nutrient solutions reported in the literature (Table 1-9). It was thought that iron or sulphur (section 1.7.7) rather than carbon may be the limiting nutrient for hydrogen production by clostridia with nutrient solution A, therefore the iron sulphate rich nutrient solution used by Fang and Liu (2002) was adapted to nutrient solution B.

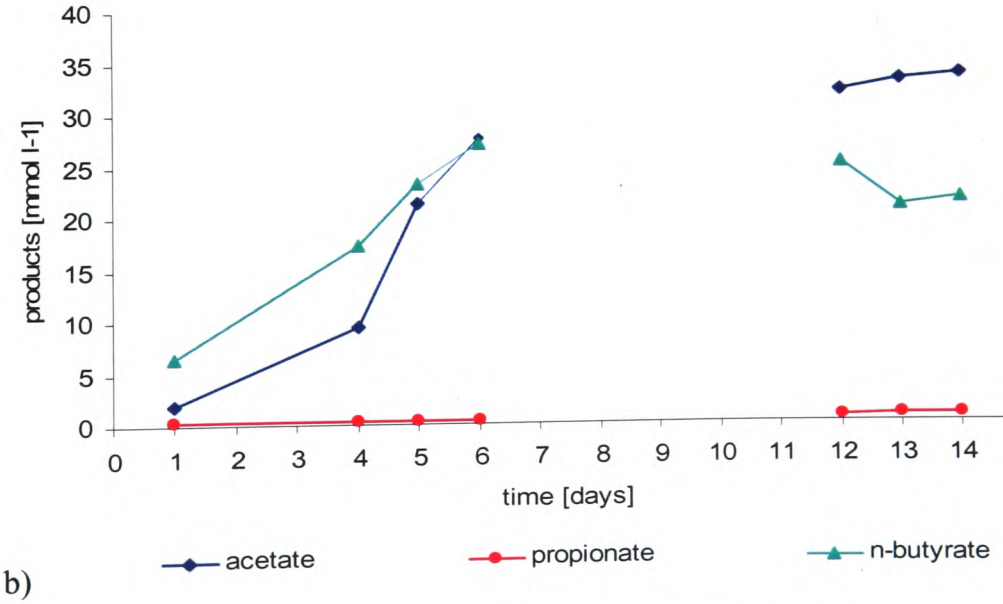
Continuous hydrogen production was achieved in experiments St2 and St3 for 14 and 8 days respectively.

### 3.1.2.1 Results of Experiment St2

Data for experiment St2 are shown in Figures 3-2 a and b. Throughout the results section day 1 in the text refers to the time between tick marks 0 and 1 on the x-axis of figures, day 2 in the text refers to the time between tick marks 1 and 2 on the x-axis and so on. Data for the batch start-up are not available, therefore the data in Figures 3-2a and b start with beginning of continuous operation at the end of day 1.




a)




b)

*Figure 3-2. Experiment St2. Continuous operation at pH 4.5, 35°C and 18 hour HRT. No data for batch start-up. Data starts hour 24, with start of cont. operation. a) gas production [ml $min^{-1}l^{-1}$] and hydrogen content [%] b) products [mmol $l^{-1}$].*

By hour 24, when continuous operation was started, an average of 1.2 ml $min^{-1}$ $l^{-1}$ gas containing 43% hydrogen was produced (Figure 3-2a). Butyrate was the main determined product with a concentration of 6.6 mmol $l^{-1}$, accompanied by acetate with a concentration of 2.0 mmol $l^{-1}$. Solvent analysis was not available.

During days 2 and 3 no off-line data was obtained. During days 4 to 14 an average of 2.1 ml $min^{-1}$ $l^{-1}$ gas with a hydrogen content of 40-50% was produced (no methane was detected). The average yields during days 4 to 6 and 12 to 14 were 1.3 and 1.0 mol hydrogen per mol hexose added respectively (residual hexose was not determined). Butyrate and acetate concentrations increased steadily, with acetate production overtaking butyrate production on day 6, when concentrations were 27.2 and 27.8 mmol $l^{-1}$ for butyrate and acetate respectively. During days 12 to 14 (VFA's were not determined during days 7 to 11) acetate was the main product with concentrations of 32.2 to 33.9 mmol $l^{-1}$, whilst the butyrate concentration decreased slightly from 25.2 to 21.8 mmol $l^{-1}$. The butyrate/acetate ratio decreased gradually during continuous operation, from 3.4 mol $mol^{-1}$ on day 1 to 0.6 mol $mol^{-1}$ on day 14. No methane or propionate was produced in this experiment, which had to be stopped due to time constraints.

### 3.1.2.2 Results of Experiment St3

Data for experiment St3 are shown in Figures 3-3a and b.

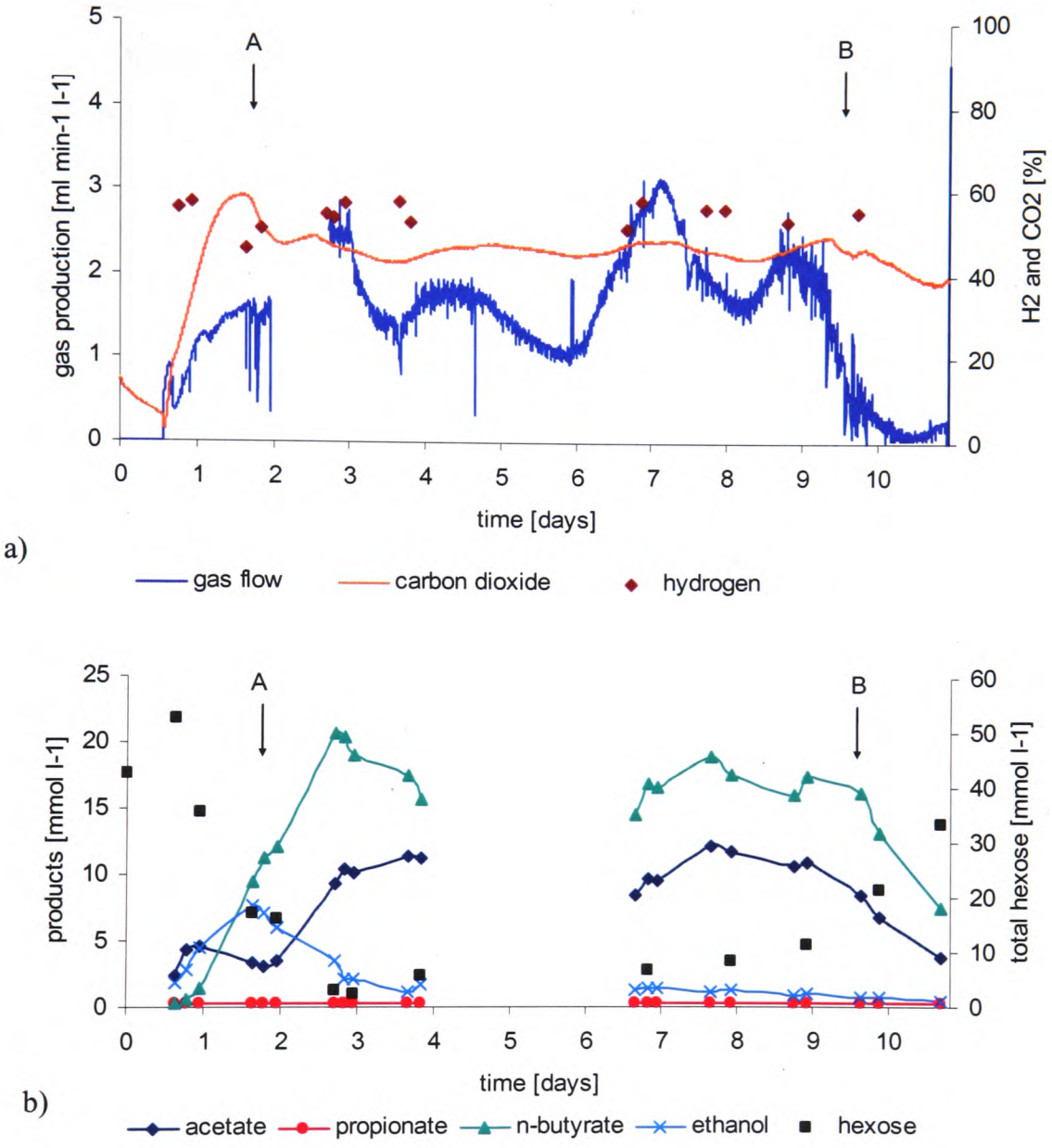



***Figure 3-3. Experiment St3. Batch start-up and continuous operation at pH 4.5, 35°C and 18 h HRT. Arrow A: continuous operation started. Arrow B: stirrer restarted. a) gas production [ml $min^{-1}$ $l^{-1}$] and composition [%] b) products and residual total hexose [mmol $l^{-1}$]***

By hour 40, the beginning of continuous operation, gas containing 52% hydrogen was produced at a rate of 1.6 ml $min^{-1}$ $l^{-1}$ (Figure 3-3a). As in experiment St2 butyrate exceeded acetate production, with concentrations of 11.3 and 3.0 mmol $l^{-1}$ respectively by hour 40 (Figure 3-3b), giving a butyrate/acetate ratio of 3.7 mol $mol^{-1}$. Solvent

analysis shows that ethanol production with a concentration of 7.0 mmol $l^{-1}$ also exceeded acetate production by hour 40.

During continuous operation gas was produced in an oscillating pattern. On day 3 gas production was not measured due to a technical problem with data transfer from the gas meter. During days 4 to 9 the average gas production was 1.8 ml $min^{-1}$ $l^{-1}$ with a hydrogen and carbon dioxide content of 55% and 46% respectively, giving an average daily yield of 1.2 mol hydrogen per mol hexose added or 1.3 mol hydrogen per mol hexose converted. Ethanol production decreased during the first 2 days of continuous operation, reaching a concentration of <2 mmol $l^{-1}$ on day 3, and remained there throughout the experiment. Butyrate remained the main non-gaseous product and was accompanied by acetate production throughout, with butyrate and acetate concentrations of 12.1 to 20.7 mmol $l^{-1}$ and 3.5 to 12.2 mmol $l^{-1}$ during days 2 to 9 respectively, whilst no propionate, acetone or butanol was detected. The butyrate/acetate ratio remained at >1.4 mol $mol^{-1}$ throughout the experiment.

After day 9 however, washout of all starch fermenting organisms was indicated by the fall in gas and acid production in combination with rising residual starch (total hexose) levels, showing the culture was susceptible to disturbances. The washout may partially have been caused by failure of the stirrer for up to 14 hours on day 10, which will have caused a decrease in pH, starvation and high hydrogen partial pressure in the bulk of the reactor, possibly leading to sporulation of clostridia. However, the gradual increase in residual hexose concentration from day 4 (Figure 3-3b) suggests that washout of the culture may have begun before the incident.

#### 3.1.2.3 Discussion of experiments St2 and St3

The hydrogen yield was similar in the two experiments: In experiment St2 the average yields during days 4 to 6 and 12 to 14 were 1.3 and 1.0 mol hydrogen per mol hexose added respectively, in experiment St3 the yield for days 4 to 9 was 1.2 mol hydrogen per mol hexose added or 1.3 mol hydrogen per mol hexose converted. Although the achieved conversion rates are far off the theoretical targets of 2 to 4 mol hydrogen per mol hexose (equations 2 and 1 respectively), they come close to rates of 1.31 and 1.36 mol hydrogen per mol hexose added in form of soluble starch, as reported by Lay (2000) from mixed heat-treated cultures at pH 4.5.

There was a remarkable difference between the product concentrations during continuous operation in the two experiments. Despite the similar hydrogen yields significantly more butyrate and acetate were produced in experiment St2 than in experiment St3. During days 12 to 14 in experiment St2 the butyrate concentration ranged from 21.8 to 25.2 mmol $l^{-1}$ compared to 14.6 to 19.0 mmol $l^{-1}$ during days 7 to 9 of experiment St3. The difference in acetate concentrations was even more apparent, ranging from 32.2 to 33.9 mmol $l^{-1}$ during days 12 to 14 in experiment St2 compared to 8.5 to 12.2 mmol $l^{-1}$ during days 7 to 9 of experiment St3. The question arises why the higher acetate and butyrate concentrations during days 12 to 14 in experiment St2 are not associated with higher hydrogen yields. If it is assumed that all acetate is produced as in equation 1 and all butyrate is produced as in equation 2, a theoretical hydrogen production rate can be calculated, which would be associated with the determined acetate and butyrate concentrations. The average acetate and butyrate concentrations were 33.2 and 22.7 mmol $l^{-1}$ during days 12 to 14 in experiment St2 and 10.5 and 16.9 mmol $l^{-1}$during days 7 to 9 of experiment St3. Given the 18 h hydraulic retention time, this converts to average acetate and butyrate production rates of 0.031 and 0.021 mmol $l^{-1}$ $min^{-1}$ during days 12 to 14 in experiment St2 and 0.010 and 0.016 mmol $l^{-1}$ $min^{-1}$ during days 7 to 9 of experiment St3. From equations 1 and 2 production of 1 mol of acetate or butyrate is associated with production of 2 mol hydrogen. Therefore theoretical hydrogen production rates of 0.104 (0.031×2 + 0.021×2) mmol $l^{-1}$ $min^{-1}$ during days 12 to 14 in experiment St2 and 0.052 (0.010×2 + 0.016×2) mmol $l^{-1}$ $min^{-1}$ during days 7 to 9 of experiment St3 in association with acetate and butyrate production can be calculated. Assuming a molar gas volume of 22.4 litres, this gives theoretical volumetric hydrogen production rates of 2.3 ml $l^{-1}$ $min^{-1}$ during days 12 to 14 in experiment St2 and 1.2 ml $l^{-1}$ $min^{-1}$ during days 7 to 9 of experiment St3. These theoretical values compare to observed hydrogen production rates of 0.9 ml $l^{-1}$ $min^{-1}$ during days 12 to 14 in experiment St2 and 1.2 ml $l^{-1}$ $min^{-1}$ during days 7 to 9 of experiment St3. The calculations show that whilst in experiment St3 the theoretical value agrees with the observed value, in experiment St2 there is a large discrepancy between the theoretical and observed hydrogen production rate. The calculations of theoretical hydrogen production rates are very simplistic, as they only consider two out of several metabolic pathways which might be present. Nevertheless they indicate that during days 7 to 9 of experiment St3 acetate and butyrate production are predominantly

or entirely associated with hydrogen production as in equations 1 and 2, whilst during days 12 to 14 in experiment St2 other metabolic pathways may be of importance. Since no methane production was detected in experiment St2, hydrogen consumption by methanogens as in equation 16 can not be the cause of the low observed hydrogen production. Equally, propionate was not detected, therefore the metabolic pathways represented by equations 14 and 15 were not present. Presence of homoacetogens on the other hand is a likely explanation for the difference between the theoretical and observed hydrogen production rates. If a proportion of the acetate was produced as in equation 10 rather than as in equation 1, this would explain why the higher acetate and butyrate concentrations during days 12 to 14 of experiment 2 were not associated with higher hydrogen yields, and why the calculations so strongly overestimated hydrogen production.

Experiments St2 and St3 therefore show that continuous hydrogen production is possible at pH 4.5, but also that hydrogen consumers may be thriving in these conditions. The decreasing butyrate/acetate ratio in experiment St2 suggests that these may gradually have become stronger during continuous operation.

Experiment St3 shows that the culture was sensitive to disturbances. It is not clear from experiments St2 and St3 if the change to nutrient solution B had any effect on hydrogen production. Although there was no indication for presence of homoacetogens in experiment St3 with nutrient solution B, there was no difference in hydrogen yields. Nutrient solution B was used for all following experiments because of its higher iron content, since iron is an essential component of ferredoxin and hydrogenase, which are both essential to hydrogen production by clostridia (Lee *et al.* 2001).

Data for a further experiment with nutrient solution A are not shown here, since technical faults during continuous operation made data interpretation difficult. However, strong propionate production was observed during continuous operation in this experiment, with a maximum concentration of 36 mmol $l^{-1}$. This shows that propionate production is possible at pH 4.5 and that the theory of Ren *et al.* (1997) -that propionic fermentation can be excluded by operation at pH 4.5- can not generally be applied.

### 3.1.3 *Continuous operation at pH 5.2 and 30°C*

Although continuous hydrogen production was achieved at pH 4.5 in experiments St2 and St3, the culture was washed out in experiment St3, and a further experiment (data not included) showed that strong propionate production was still possible. Most successful hydrogen production is reported at pH 5.2 to 5.5 (Table 1-2). Lay (2000) showed through statistical analysis of 23 experiments on soluble starch that pH 5.2 allowed maximum hydrogen production (decreasing by over 10% at pH <4.7). Hence the pH was changed to 5.2 for experiments St4, St5 and St6.

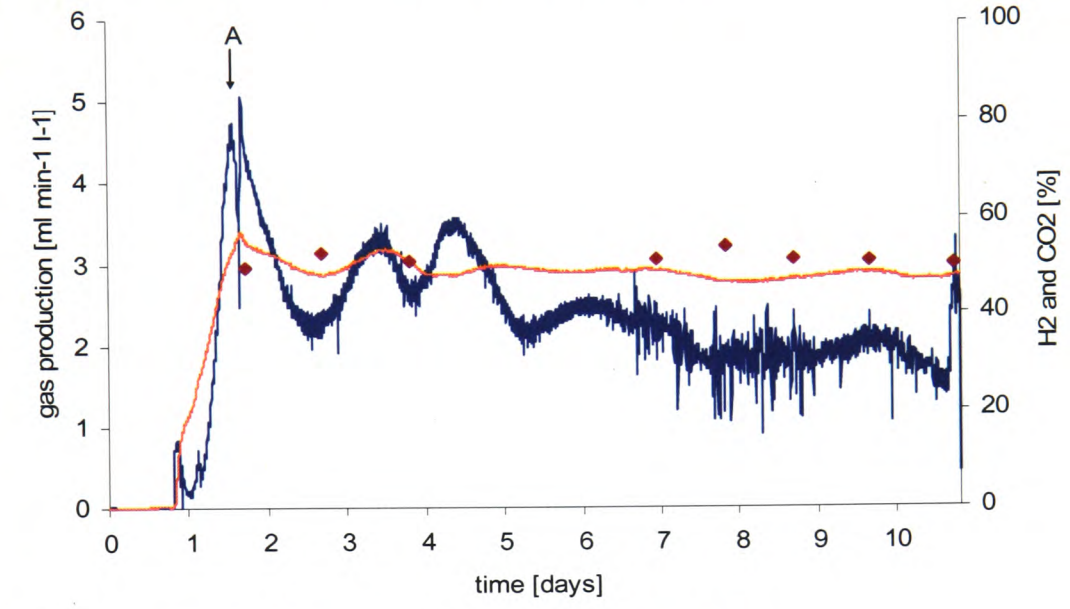
For experiments at pH 5.2 the temperature was changed from 35°C in experiments at pH 4.5 to 30°C, because Zoetemeyer *et al.* (1982a) reported from an acidogenic reactor on glucose at pH 5.8, inoculated with activated sludge, that for 10 h HRT minimum butyrate and maximum propionate production was observed at 35°C, when operating temperatures between 20°C to 40°C were tested. At 30°C in comparison buytrate was the main product and propionate production low. Although hydrogen production was not analysed in these experiments, it was thought that the butyrate production was most likely connected to hydrogen production. It was thus concluded that 30°C might be more suitable for hydrogen production, and would reduced energy input.

It was thought possible that clostridia, which are assumed to be the main hydrogen producers in the culture, sporulate in adverse conditions, for example in experiment St3, when the stirrer failed. This can be of great disadvantage for the spore formers, since they may be washed out and/or out-competed for substrate, if conditions favour the vegetative cells of non-spore formers. In previous experiments with glucose as sole carbon source and an enrichment culture as inoculum (Mizuno *et al.* 2000a), reactivation of a hydrogen producing culture after partial washout was possible within 2 days (Dinsdale 2001). 1 g $l^{-1}$ glucose was added in experiments St4, St5 and St6, as it was thought that this may prevent sporulation / encourage germination in clostridia and therefore make the culture more resilient. Also, 1 g $l^{-1}$ peptone was added in experiments St4, St5 and St6, because Yokoi *et al.* (2001) found it an essential addition to sweet potato starch in a pure culture of *Clostridium butyricum*.

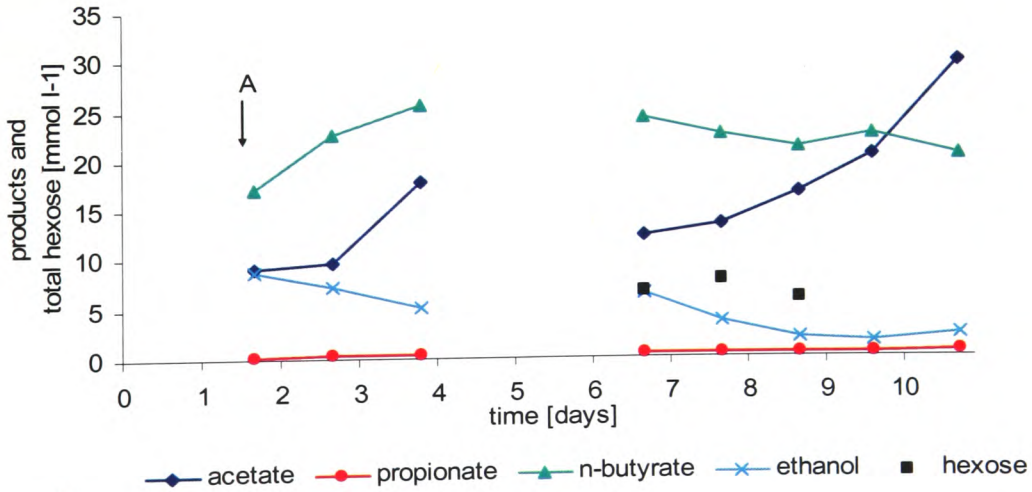
Hydrogen was produced during continuous operation in all three experiments (St4, St5 and St6) at pH 5.2, but to varying degrees. The most stable hydrogen production was achieved in experiment St4.

### 3.1.3.1 Experiment St4

Data for experiment St4 are shown in Figures 3-4a and b.




a)




b)

***Figure 3-4. Experiment St4. Batch start-up and continuous operation at pH 5.2, 30°C and 18 h HRT. Arrow A: Continuous operation started. a) gas production [ml $min^{-1}$ $l^{-1}$] and composition [%] b) products and residual total hexose [mmol $l^{-1}$].***

During batch start-up gas production reached a maximum of 4.7 ml $min^{-1}$ $l^{-1}$ at hour 37 (Figure 3-4a). At hour 40, when continuous operation was started, the gas contained 49% hydrogen. Butyrate was the main determined product with a concentration of 17.2

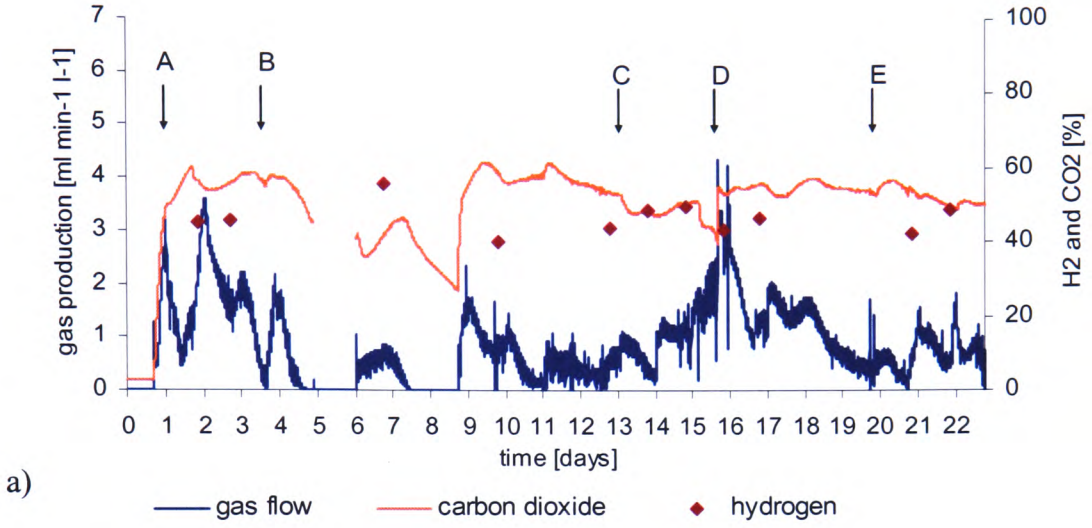
mmol $l^{-1}$, accompanied by acetate and ethanol in concentrations of 9.2 and 8.7 mmol $l^{-1}$ (Figure 3-4b), giving a butyrate/acetate ratio of 1.9 mol $mol^{-1}$.

Figure 3-4a shows that hydrogen production oscillated strongly during the first 5 days of continuous operation. The average gas production rate during days 7 to 9, when gas production was not oscillating, was 2.1 ml $min^{-1}$ $l^{-1}$. The produced gas consisted entirely of carbon dioxide and hydrogen, with a hydrogen content of 45 to 55%, giving an average yield of 1.3 mol hydrogen per mol hexose converted during days 7 to 9. No methane was detected.

As in experiment St3 ethanol was mainly produced during start-up (Figure 3-4b), while during continuous operation the concentration gradually decreased to below 2 mmol $l^{-1}$. Throughout the experiment no propionate, acetone or butanol was detected. During days 2 to 10 more butyrate (17.2 to 25.6 mmol $l^{-1}$) than acetate (9.2 to 20.5 mmol $l^{-1}$) was produced (Figure 3-4b). However, during days 7 to 11 the acetate concentration more than doubled (from 12.3 to 30.1 mmol $l^{-1}$). Overall the butyrate/acetate ratio decreased from 1.9 mol $mol^{-1}$ at the beginning of continuous operation to 0.7 mol $mol^{-1}$ on day 11. Similar to the observations made from comparison of experiments St2 and St3, the increase in hydrogen with acetate production as expected from equation 1 was not observed: hydrogen and butyrate production varied little between days 7 and 11, with an average daily yield of 1.1± 0.1 mol hydrogen per mol hexose added and butyrate concentrations between 20.6 and 22.7 mmol $l^{-1}$. As in experiment St2, it is possible that the homoacetogenic metabolism described by equations 10 to 13 was developing, either due to a metabolic shift of which the clostridia are capable (Rogers and Gottschalk 1993) or due to the development of a population of non clostridial homoacetogens. The experiment had to be ended due to time constraints.

### 3.1.3.2 Experiment St5

Data for experiment St5 are shown in Figures 3-5a and b.



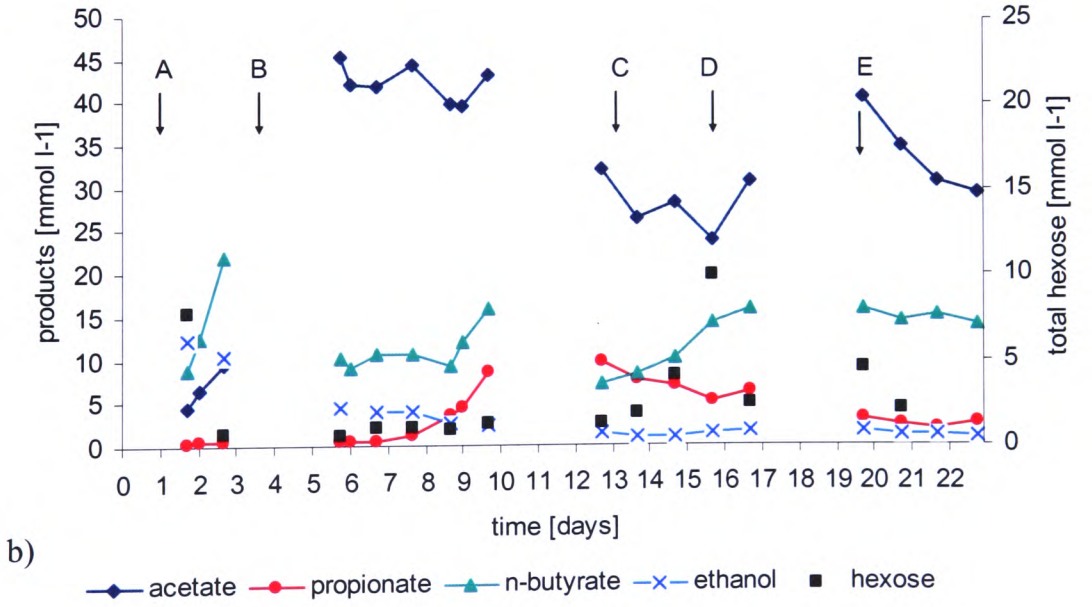




***Figure 3-5. Experiment St5. Batch start-up and continuous operation at pH 5.2, 30°C, 18, 12 and 9 h HRT. Arrow A: continuous operation started. Arrow B: starch supply re-instated. Arrow C: HRT decreased to 12 h. Arrow D: stirrer restarted. Arrow E: HRT decreased to 9 h. a) gas production [ml $min^{-1}$ $l^{-1}$] and composition [%] b) products and residual total hexose [mmol $l^{-1}$].***

Gas production during batch start-up reached a maximum of 3.4 ml $min^{-1}$ $l^{-1}$ at hour 24, when continuous operation was started. No offline data was obtained during batch start-up.

During the first two days of continuous operation (days 2 and 3) butyrate exceeded acetate concentration with 22.0 mmol $l^{-1}$ and 9.4 mmol $l^{-1}$ respectively on day 3 (Figure3-5b). The gas production rate varied strongly during this period, from 0.5 to 3.5 ml $min^{-1}$ $l^{-1}$ (Figure3-5a), with a hydrogen content of 45%, giving a yield of 0.9 mol hydrogen per mol hexose converted on day 3. As in experiments St3 and St4, ethanol, which was the dominant product on day 2 with a concentration of 12.1 mmol $l^{-1}$, decreased gradually throughout continuous operation (Figure3-5b).

Between days 3 and 6 the dominant metabolism changed strongly (Figure3-5b): the butyrate concentration decreased from 20.8 mmol $l^{-1}$ on day 3 to 10.1 mmol $l^{-1}$ on day 6, whilst the acetate concentration increased from 9.3 to 45.3 mmol $l^{-1}$. This may be caused by disturbances due to technical problems on day 4: during the night the starch supply failed (up to 16 hours interruption) and the stirrer head had loosened, thus the rate of stirring decreased. After reinstatement of starch supply and stirring, gas production increased rapidly to around 2 ml $min^{-1}$ $l^{-1}$ on day 4 and then ceased for 30 hours (days 5 and 6).

During days 6 to 9, when acetate concentrations remained high (39.5 to 45.4 mmol $l^{-1}$) and butyrate concentration low (8.9 to 15.8 mmol $l^{-1}$) (Figure3-5b), hydrogen production was negligible (Figure3-5a; av. yield on day 7 was 0.3 mol hydrogen per mol hexose converted). Following this occurrence of strong acetate production without coinciding hydrogen production, propionate production began on day 8, reaching a concentration of 8.5 mmol $l^{-1}$ on day 13. Daily average hydrogen yields on days 7, 10 and 13 (the only days when there was sufficient gas production to determine hydrogen concentration) were 0.3, 0.3 and 0.2 mol hydrogen per mol hexose converted respectively. Although a clear correlation between low hydrogen yield and propionate production could not be drawn due to the strong variations in gas, acetate and butyrate production, it can be concluded from equation 15 that the two were most likely connected.

Since pH has been shown to have little effect on propionate production, which was observed at pH 4.5 (data not shown), pH 5.2 (experiment St4) and pH 6.0 (data not shown), the HRT was shortened to 12 h on day 13 to investigate if this would disadvantage propionate producers, since the genus Propionibacterium is known to be slow growing (section 1.3.4). At the shorter retention time of 12 h the propionate concentration decreased gradually, from 7.7 mmol $l^{-1}$ on day 14 to 3.0 mmol $l^{-1}$ on day 20, whilst butyrate production and hydrogen yields increased slightly, from 0.3 to 0.5 mol hydrogen per mol hexose converted during days 14 to 17. The acetate concentration decreased during days 14 to 16, but increased back to 40.8 mmol $l^{-1}$ during days 16 to 20, possibly a consequence of a stirrer failure during the night of day 15.

On day 20 the retention time was shortened further to 9h. At 9h HRT the acetate concentration decreased from 40.8 mmol $l^{-1}$ on day 20 to 29.5 mmol $l^{-1}$ on day 23, whilst butyrate and propionate concentrations stayed constant at 14.1 to 15.9 and 1.8 to 3.0 mmol $l^{-1}$ respectively. The average daily yield on days 21 and 22 was very low with 0.1 and 0.2 mol hydrogen per mol hexose converted. The reduction in acetate production may indicate that the short retention time disadvantages homoacetogens, but despite the steady butyrate production this did not increase hydrogen yields. Experiments St5 therefore indicates that shortening of the HRT to 12 hours possibly disadvantages propionate producers, but does not clearly decrease or increase hydrogen production directly.

### 3.1.3.3 Experiment St6

In experiment St6, as in experiment St5, hydrogen production was highly variable with lower overall yields than in experiment St4. The gas production rate during batch start-up reached an unusually high maximum of 11 ml $min^{-1}$ $l^{-1}$ at hour 30 and then decreased to 0.2 ml $min^{-1}$ $l^{-1}$ by hour 40 (Figure3-6a), when continuous operation was started.

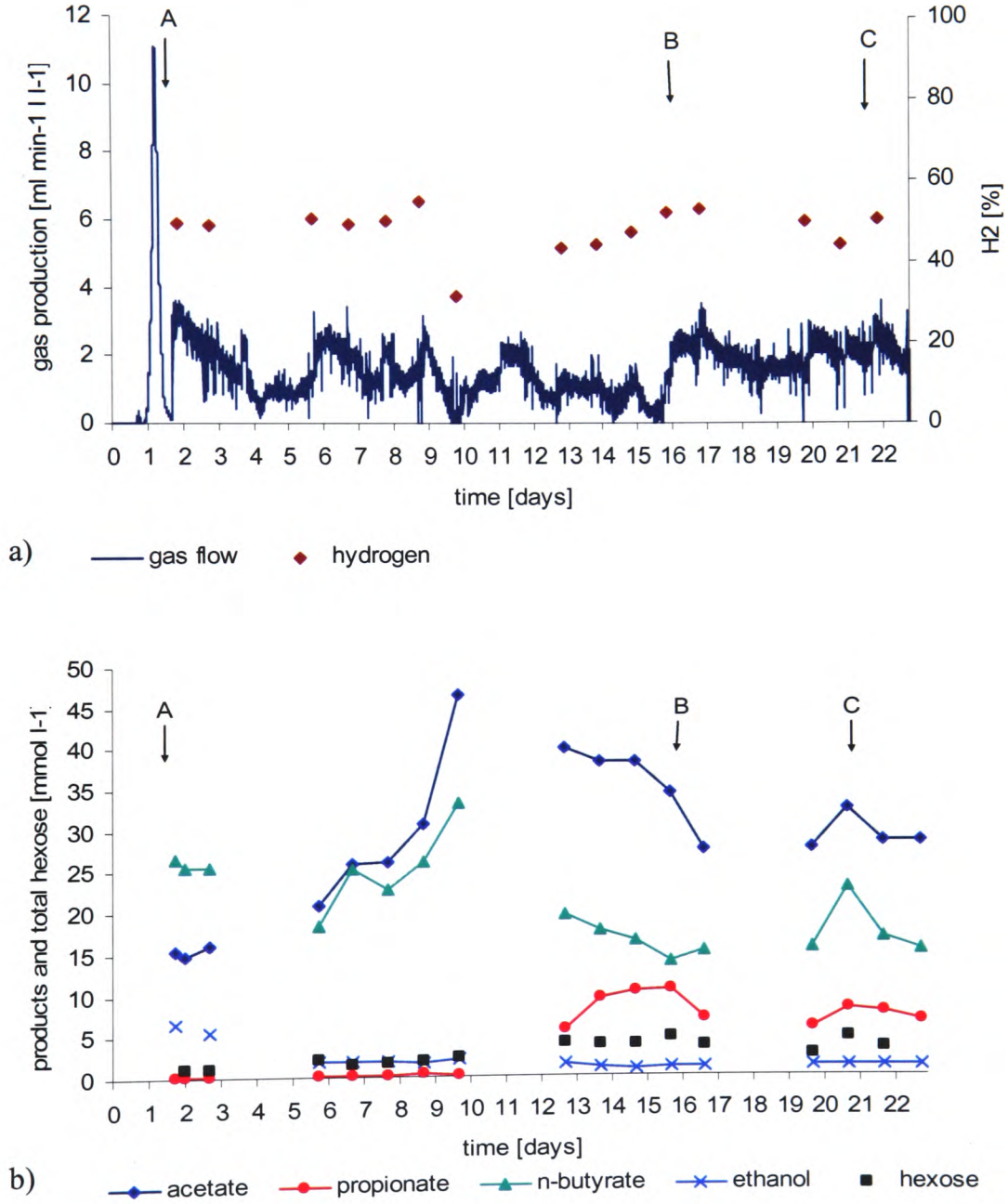



***Figure 3-6. Experiment St6. Batch start-up and continuous operation at pH 5.2, 30°C, 18 and 12 h HRT. Arrow A: continuous operation started. Arrow B: HRT decreased to 12 h. Arrow C: water supply unblocked. a) gas production [ml $min^{-1}$ $l^{-1}$] and hydrogen content [%] b) products and residual total hexose [mmol $l^{-1}$].***

As in experiment St4 butyrate was the main product during batch start-up with a concentration of 26.6 mmol $l^{-1}$ at hour 40, accompanied by acetate and ethanol at concentrations of 15.4 and 6.5 mmol $l^{-1}$ respectively (Figure3-6b). No propionate, acetone or butanol was detected. All substrate was consumed before continuous operation was started.

With the beginning of continuous operation gas production started at a rate of 3 ml $min^{-1}$ $l^{-1}$ and a hydrogen content of 49%, giving a yield of 1.3 mol hydrogen per mol hexose converted on day 3. This shows that here hydrogen production could be reactivated instantly after a short interruption in feed supply. Between days 3 and 6 the butyrate concentration decreased from 25.6 to 18.4 mmol $l^{-1}$, whilst the acetate concentration increased from 16.0 to 20.8 mmol $l^{-1}$, making acetate the main product as after day 3 of experiment St5. Ethanol production decreased in continuous operation, and concentrations remained below 2 mmol $l^{-1}$ throughout the experiment.

During days 6 to 10 butyrate and acetate concentrations increased steeply, from 18.4 to 33.3 mmol $l^{-1}$ and from 20.8 to 48.0 mmol $l^{-1}$ respectively, whilst a corresponding increase in hydrogen yield was not observed (av. daily yields of 0.5, 1.0, 1.0, 0.9 and 0.6 mol hydrogen per mol hexose converted on days 6 to 10 respectively). The butyrate/acetate ratio, which had decreased from 1.7 on day 2 to 0.9 on day 6, decreased further to 0.7 on day 10.

As in experiment St5, the occurrence of strong acetate production without coinciding hydrogen production was followed by the onset of propionate production after day 10, reaching a concentration of 9.5 mmol $l^{-1}$ on day 14. Therefore the same problems as in experiment St5 –dominant acetate production and presence of propionate producers- were encountered during days 13 to 16 of experiment St6. Hydrogen yields were accordingly low, with daily average yields of 0.5 to 0.8 mol hydrogen per mol hexose converted.

On day 16 the HRT was shortened to 12 h, which caused a rather slight and gradual decrease of propionate concentration to from 10.5 mmol $l^{-1}$ on day 16 to 6.8 mmol $l^{-1}$ on day 23 and a small increase in daily average yield from 0.5 (day 17) to 0.8 (day 22) mol

hydrogen per mol hexose converted. As in experiment St5, the shortening of the retention time to 12 h did not have a clear effect on hydrogen production, but possibly restricted propionate production.

## *3.2 Experiments with nitrogen sparging*

In the 3 experiments described here it was investigated if continuous hydrogen production could be improved by reduction of the hydrogen partial pressure through sparging with nitrogen. It was proposed that sparging with nitrogen should increase hydrogen yields through stripping of hydrogen from the reactor liquid, thus preventing reduced end-product formation (propionate and ethanol) and hydrogen consumption, and reducing loss of hydrogen dissolved in the effluent.

### *3.2.1 Experiment St7*

Data for experiment St7 is shown in Figure 3-7. Butyrate was the main product during batch start-up, with a concentration of 24.4 mmol $l^{-1}$ at hour 42, when continuous operation was started (Figure 3-7b). The batch hydrogen yield for this experiment was 1.5 mol $mol^{-1}$ hexose consumed. Hydrogen and carbon dioxide production rates [ml $min^{-1}$ $l^{-1}$] were calculated from continuous measurements of total gas and on- or offline measurement of its carbon dioxide or hydrogen content respectively.

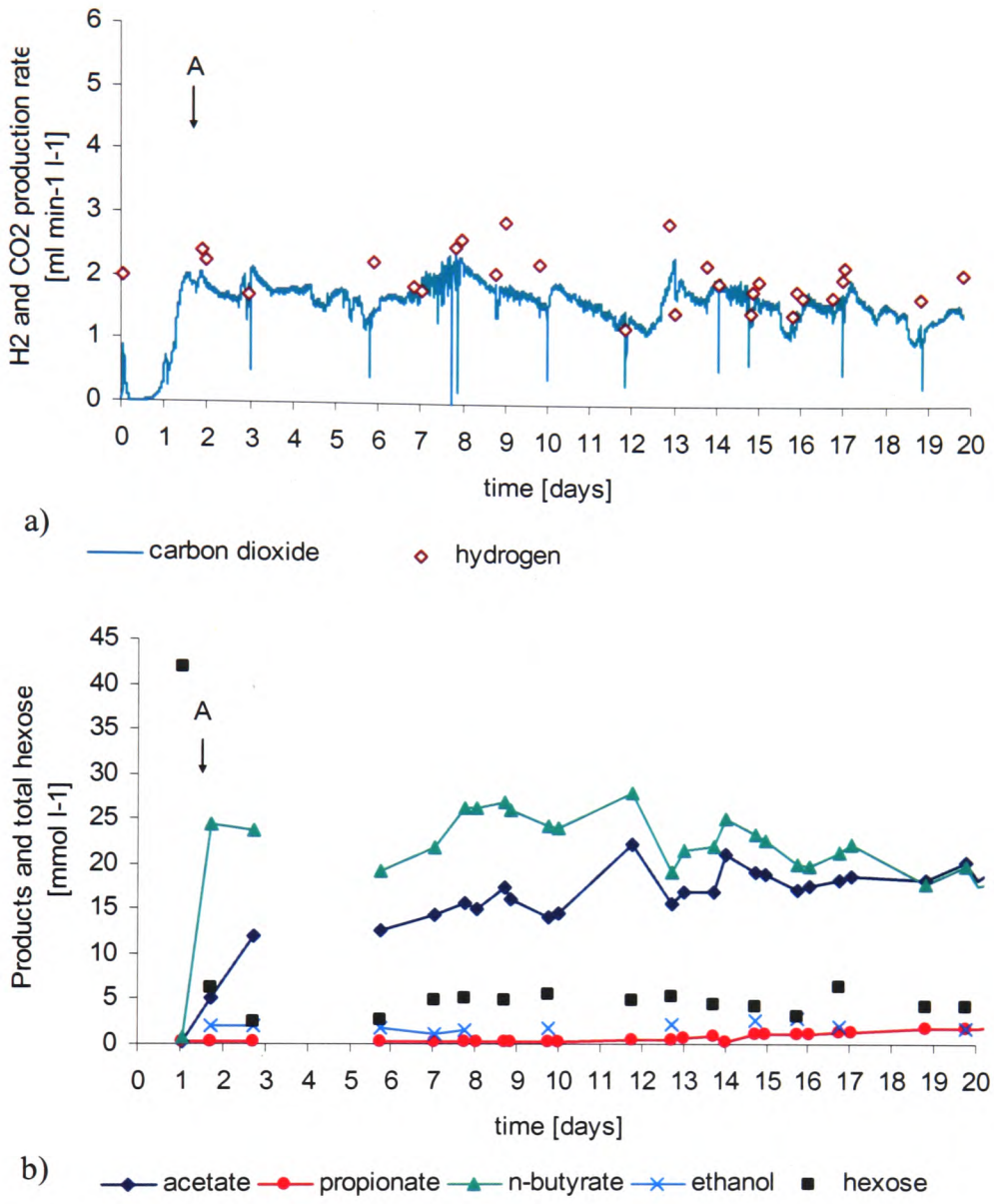



***Figure 3-7. Experiment St7. Batch start-up and continuous operation with sparging at pH 5.2, 32°C and 15 h HRT. Arrow A: continuous operation started. a) hydrogen and carbon dioxide production rates [ml $min^{-1}$ $l^{-1}$] b) products and residual total hexose [mmol $l^{-1}$].***

During continuous operation hydrogen production did not oscillate and was less variable than in experiments St2 to St6 without sparging (see Figures 3-2 to 3-6). The average total gas flow after 3 HRTs of continuous operation was 66.3 ± 3.0 ml $min^{-1}$, containing approximately 6.9 % hydrogen (data not shown), giving an average hydrogen production rate of 2.0 ml $min^{-1}$ $l^{-1}$ (Figure 3-7a) during days 6 to 20. The average yield during days 6 to 20 was 1.9 mol hydrogen $mol^{-1}$ hexose converted or 1.7 mol hydrogen $mol^{-1}$ hexose added. Butyrate was the main product until day 19, with concentrations of 18.0 to 28.0

mmol $l^{-1}$ (Figure 3-7b). Acetate concentrations increased gradually during continuous operation from 12.0 mmol $l^{-1}$ on day 3 to 18.0 mmol $l^{-1}$ on day 19. Propionate production was present from day 12 but did not increase to concentrations over 2.0 mmol $l^{-1}$. The experiment was ended on day 19 because the hard-drive of the online monitoring system failed. The average hydrogen yield of 1.7 mol per mol hexose added during 15 days in this experiment (pH 5.2, 15 h HRT and 32°C), was significantly higher than the optimum yield of 1.3 mol per mol hexose added achieved without sparging in experiments St2 and St3 (pH 4.5, 18 h HRT and 35°C) and St4 (pH 5.2, 18 h HRT and 30°C). If the increase in yield is due to the lowered hydrogen partial pressure, it could possibly be suppressed further by technically improved sparging procedures.

### *3.2.2 Experiment St8*

For experiment St8 the feed concentration was increased to 20 g $l^{-1}$ to investigate the effect of higher substrate concentration on hydrogen yield and substrate conversion efficiency. If substrate conversion rates and hydrogen yields equal to experiment St7 could be achieved with higher substrate concentrations, this would improve the overall energy balance of the system significantly, as a given increase in the feed concentration would not require the same increase in energy input to reactor heating, stirring and pumping. To date maximum concentration of a particulate substrate for hydrogen production has not been investigated.

To allow direct comparison with results from experiment St7 operating parameters were not changed with exception of the following alterations:

- As described in section 2.7.1.2 a sinterstick of defined pore size giving smaller bubbles was used for sparging
- glucose was not added, because there was no evidence from experiments St1 to St7 that it improved hydrogen production, and because it was noticed that Mitchell (1998) report incidences where decomposition of starch by some clostridial strains was discouraged by addition of soluble sugars (section 1.7.2).
- The sludge inoculum was heated to 110°C, since these experiments were performed after the changes in Cog Moors sewage works described in section 2.5.

In addition to measurements in experiment St7, the redox and online hydrogen sensor were fitted, and lactate and formate were determined off-line.

Data for experiment St8 are shown in Figure 3-8. As stated in section 2.7.1.2, hydrogen and carbon dioxide data for experiments with sparging are displayed in the form of production rates [ml $min^{-1}$ $l^{-1}$], which were calculated from the total gas flow out [ml $min^{-1}$ $l^{-1}$] and its hydrogen/carbon dioxide content [%].

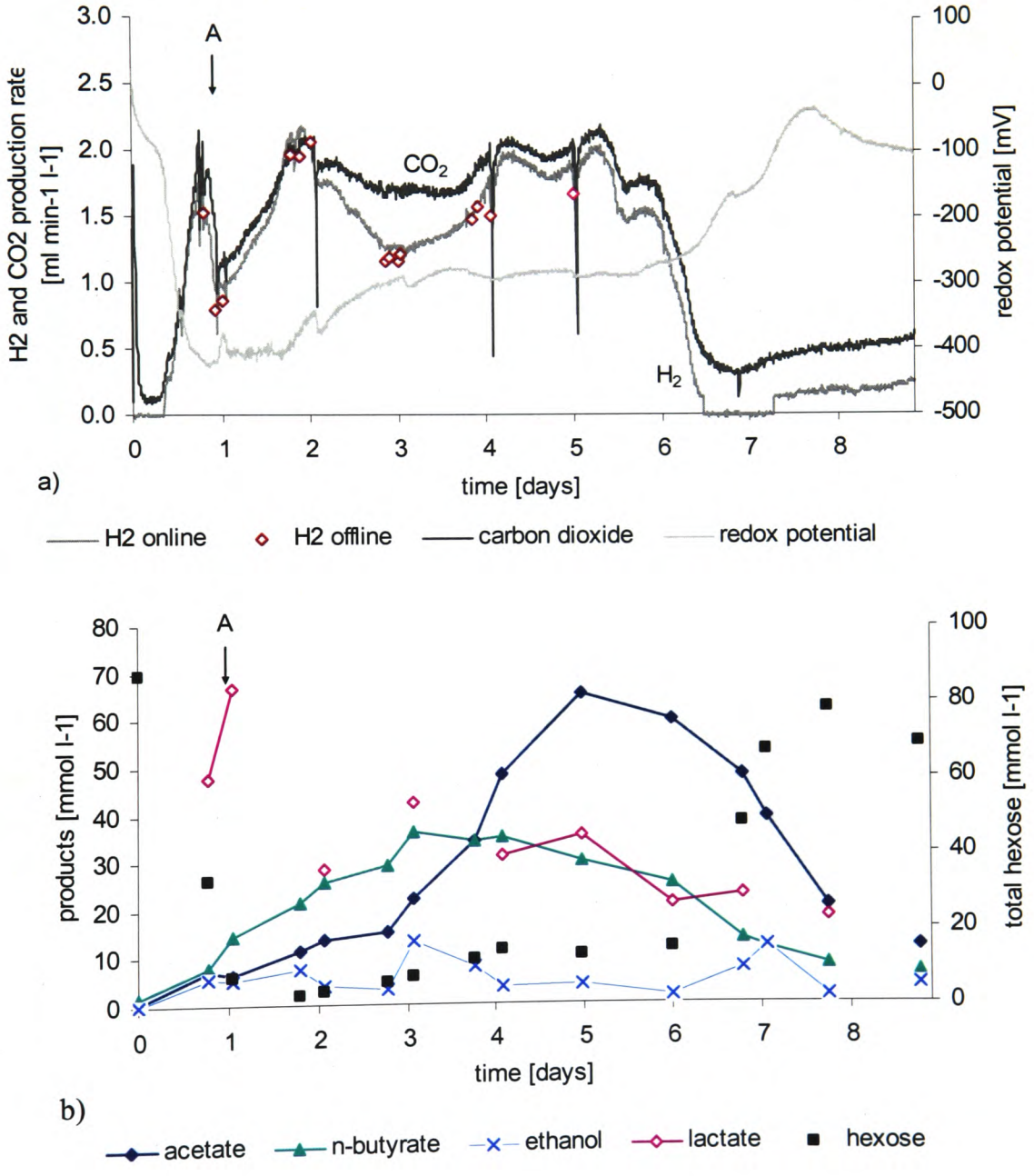



***Figure 3-8. Experiment St8. Batch start-up and continuous operation at 20g $l^{-1}$ starch, pH 5.2, 32°C and 15 h HRT with $N_2$ sparging. Arrow A: continuous operation started. a) hydrogen and carbon dioxide production rates [ml $min^{-1}$ $l^{-1}$] and redox potential [mV] b) products and residual total starch [mmol $l^{-1}$].***

Figure 3-8a shows online data including the added redox and hydrogen sensor data. Figure 3-9 shows key periods for redox potential changes on a smaller scale.

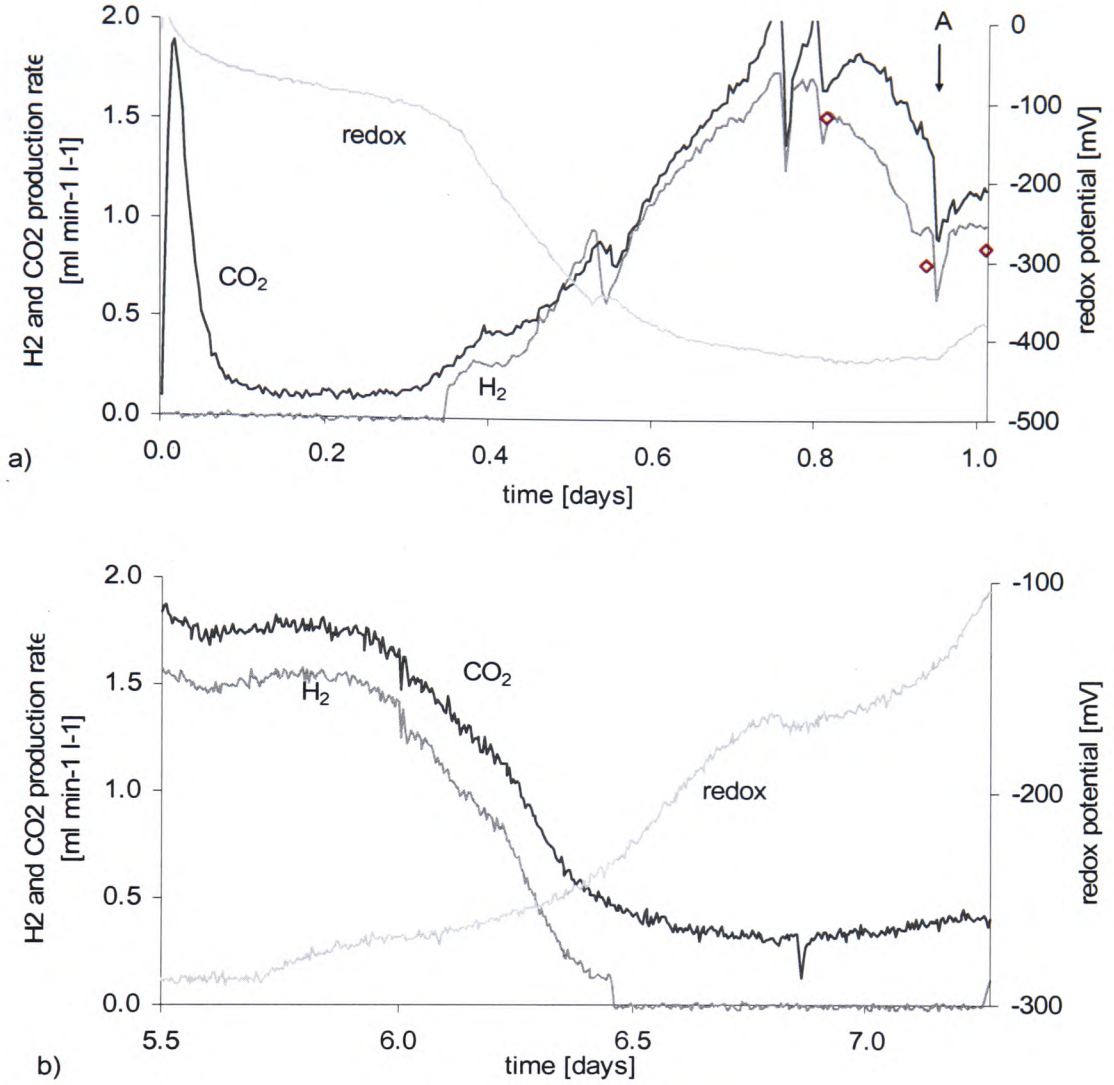



***Figure 3-9. Hydrogen and carbon dioxide production [ml $min^{-1}$ $l^{-1}$] and redox potential [mV] during selected periods of experiment St8. Arrow A: continuous operation started. a) day 1 b) period of washout.***

Figures 3-8a and 3-9a show that in experiment St8 the redox potential decreased sharply from the start and had reached -130mV by hour 8 (day 0.33), when hydrogen production was first detected. This indicates microbial activity that did not involve measurable gas production but consumed oxygen. During batch start-up the hydrogen production rate reached a peak of 1.7 ml $min^{-1}$ $l^{-1}$ at hour 18 (day 0.75) and then decreased to around 1.0 ml $min^{-1}$ $l^{-1}$, giving a batch yield of 0.6 mol hydrogen per mol hexose converted. By hour 23 (day 0.96), when continuous operation was started, the redox potential had decreased to –425mV. Lactate was the main product with a concentration of 66.8 mmol

$l^{-1}$, accompanied by 14.6 mmol $l^{-1}$ butyrate, 6.4 mmol $l^{-1}$ acetate and 5.0 mmol $l^{-1}$ ethanol.

During the first 5 days of continuous operation (= 8 HRTs) the hydrogen production rate oscillated from 1 to 2 ml $min^{-1}$ $l^{-1}$ (Figure 3-8a), whilst the redox potential increased gradually to –280 mV. The largest decrease in hydrogen production, which occurred on day 3, was associated with an increase in redox potential from -370 mV to -290 mV.

The substrate conversion decreased slightly from 97% on the first day of continuous operation to 85% on day 5 (Figure 3-8b). The lactate concentration decreased during the first day of continuous operation and was then similar to the butyrate concentration. Butyrate exceeded acetate concentrations until day 3, when butyrate reached a maximum of 36.3 mmol $l^{-1}$ and decreased steadily thereafter, whilst the acetate concentration continued to increase to a maximum of 65.7 mmol $l^{-1}$ on day 5. Daily average hydrogen yields were with 0.8 mol per mol hexose converted equal on days 2 and 5. Whilst average daily butyrate concentrations were with 27. 4 and 27.6 mmol $l^{-1}$ very similar on days 2 and 5, the average daily acetate concentration was 14.4 mmol $l^{-1}$ on day 2 compared to 63.0 mmol $l^{-1}$ on day 5. As proposed in section 3.1.2.3 for experiment St2, this may indicate development of homoacetogenesis in experiment St8.

From day 6 hydrogen and carbon dioxide production decreased rapidly (Figure 3-8a), and hydrogen production ceased on day 7. This coincided with an increase in residual total starch to 47.6 mmol $l^{-1}$, reducing starch conversion to 53%, and a decrease in acetate and butyrate concentrations to 48.4 and 13.7 mmol $l^{-1}$ respectively. The decrease in gas production also coincided with an increase in redox potential (Figure 3-9b). However, the redox potential increased only from -290 mV to -240 mV by the time the hydrogen production ceased, but increased further to –40mV thereafter (Figures 3-8a and 3-9b). Therefore the bulk of the increase in redox potential followed rather than coincided with the decline in hydrogen production. During the remaining 2 days of the experiment the hydrogen production did not increase past 0.25 ml $min^{-1}$ $l^{-1}$. Since product concentrations decreased further, whilst residual total starch concentrations increased, the culture was probably washing out. The cause for this is unknown since there were no changes made during continuous operation and no technical problems were encountered.

### 3.2.3 *Experiment St9*

Data for experiment St9 are shown in Figure 3-10. The experiment was a repeat of experiment St8. The aim was to investigate whether the duration of hydrogen production could be extended and the yield increased.

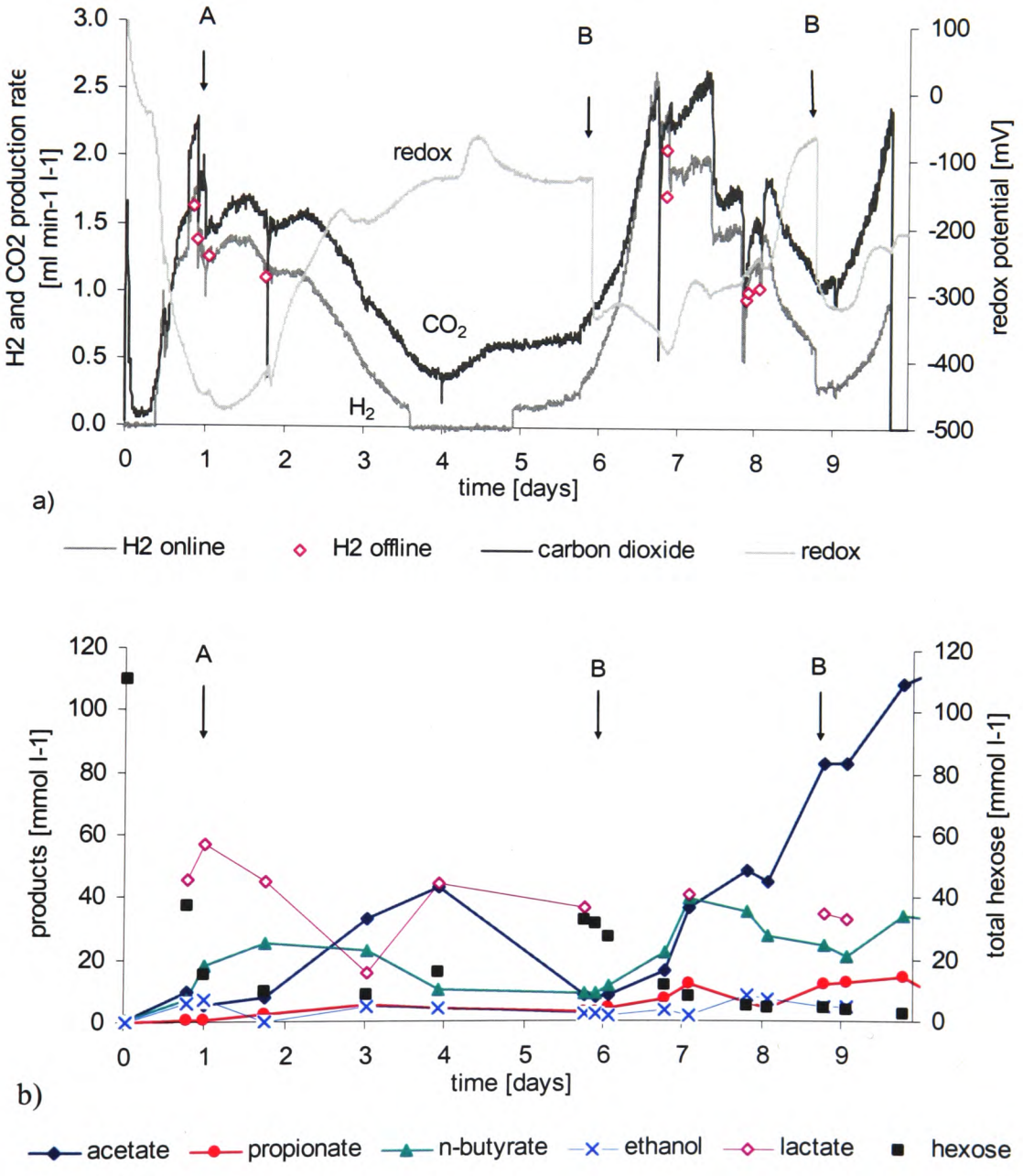



***Figure 3-10. Experiment St9. Batch start-up and continuous operation at 20 g $l^{-1}$ starch, pH 5.2, 32°C and 15 h HRT with $N_2$ sparging. Arrow A: continuous operation started. Arrow B: cysteine injected a) hydrogen and carbon dioxide production rates [ml $min^{-1}$ $l^{-1}$] and redox potential [mV] b) products and residual total starch [mmol $l^{-1}$].***

Figure 3-11 shows key periods for redox potential changes on a smaller scale.

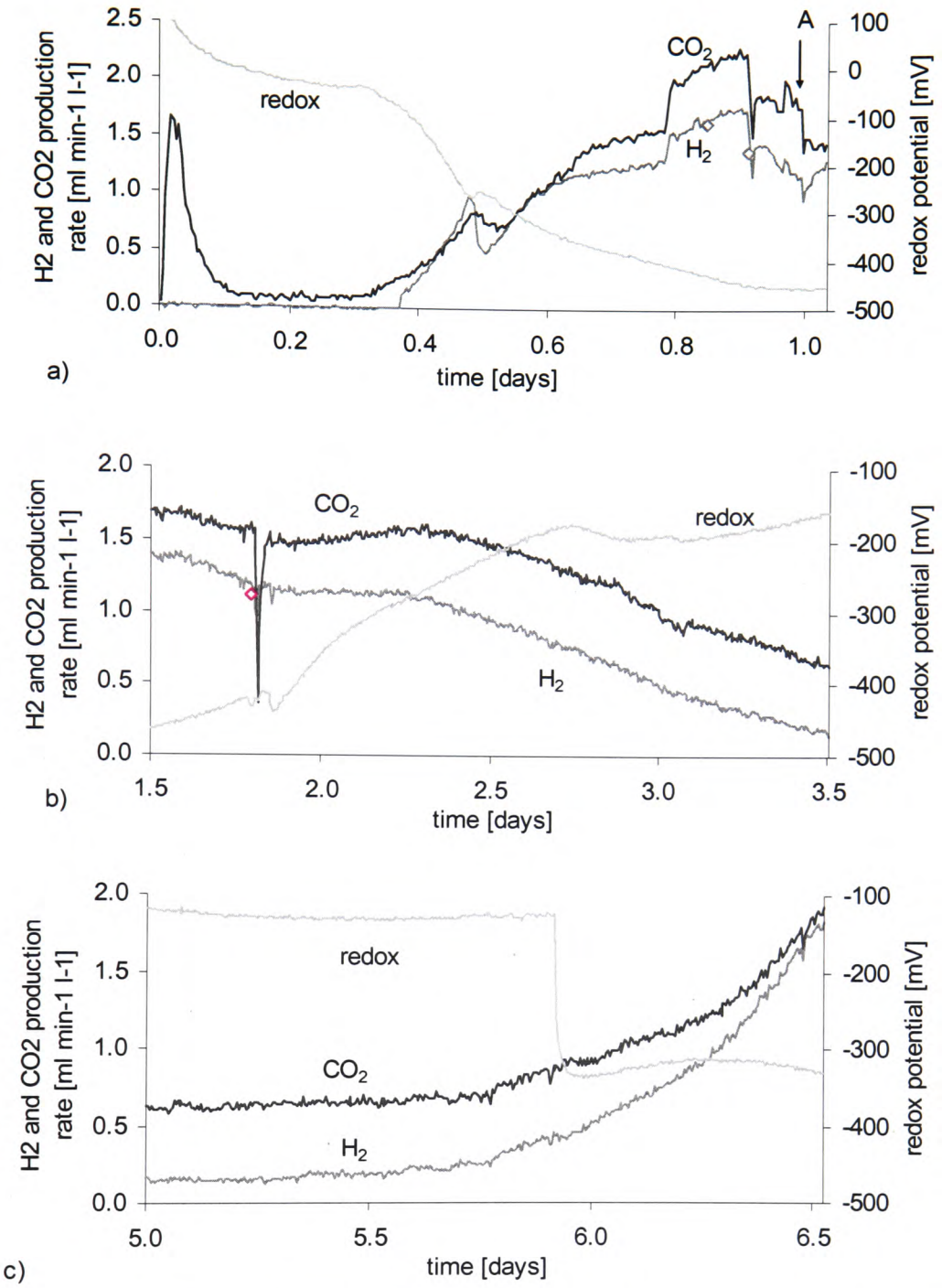



***Figure 3-11. Hydrogen and carbon dioxide production [ml $min^{-1}l^{-1}$] and redox potential [mV] during selected periods of experiment St9. Arrow A: continuous operation started. a) day 1 of experiment b) period of washout c) first cysteine injection.***

The redox potential at the very beginning (time 0) of experiment St9 was approximately 100 mV higher than that at the very beginning of experiment St8 (Figures 3-9a and 3-9c). As in experiment St8 the redox potential decreased from the start and reached –80 mV by hour 8 (day 0.33), when hydrogen production started (Figures 3-9c and 3-10a). During batch start-up the hydrogen production rate reached a peak of 1.7 ml $min^{-1}$ $l^{-1}$ at hour 21 and then decreased to around 1.2 ml $min^{-1}$ $l^{-1}$, giving a batch yield of 0.5 mol

hydrogen per mol hexose converted. By hour 24, when continuous operation was started, the redox potential had decreased to –450 mV (similar to the minimum of -425 mV in experiment St8) and, as in experiment St8, lactate was the main product with a concentration of 57.1 mmol $l^{-1}$ (Figure 3-10b). As before, butyrate concentration exceeded acetate and ethanol concentrations with 17.7 mmol $l^{-1}$ compared to 5.1 and 7.5 mmol $l^{-1}$ respectively.

Hydrogen production of > 1 ml $min^{-1}$ $l^{-1}$ only continued for the first two retention times, then started to decrease on day 3 and ceased on day 4 (Figure 3-10a). As in experiment St8 the decrease in gas production was associated with an increase in redox potential. Unlike the sequence of events observed at the end of experiment St8, here the redox potential increased mainly prior to the decrease in gas production (Figure 3-9d). At the point when hydrogen production started to decrease rapidly, the redox potential had already increased from a minimum of -470 mV to –240mV. On day 3 of experiment St8 a small decrease in hydrogen production had also been observed, but there the culture recovered during day 4, whilst here hydrogen production ceased for 2 retention times. Whilst the sequence of events differs in the two experiments, the scale of redox changes is similar in the two experiments. In experiment St8 the redox potential had increased from -420 mV on day 2 to -270 mV at the point when gas production started to decrease on day 6. This compares to an increase in experiment 9 from -470 mV on day 2 to -240 mV at the point when gas productions started to decrease on day 3. This may suggest that there is a maximum redox value, here around -240 to -270 mV, which either indicates or causes failure of hydrogen production.

Whilst the butyrate concentration decreased with decreasing hydrogen production to 10.1 mmol $l^{-I}$, acetate and lactate concentrations increased from 33.2 and 15.8 mmol $l^{-1}$ on day 3 to 43.4 and 44.4 mmol $l^{-1}$ respectively on day 4 (Figure 3-10b). This indicates that a possible cause for the decrease in hydrogen production was a change in metabolism and/or microbial population from butyrate/hydrogen to acetate and lactate producing. This change in metabolism is possibly linked to the increase in redox potential, but if so, it is not clear from the available data which would be the cause and which the effect.

On day 6 lactate was the main product detected. Butyrate, ethanol and propionate production was low, the acetate concentration decreased from 43.4 on day 4 to 7.7 mmol $l^{-1}$, residual starch levels increased and the redox potential remained high at around –120 mV. Despite these indicators of unfavourable conditions hydrogen production started again on day 5. On day 6 L-cysteine was injected into the reactor to a concentration of 0.1%, to investigate if lowering of the redox potential would encourage hydrogen production. The injection caused an instant drop in redox potential from –120mV to –330mV and was associated with a more gradual increase in hydrogen and carbon dioxide production (Figure 3-9e). However, this increase had already started before L-cysteine was injected (this had not been detected on the day), therefore it can not be concluded that the addition of L-cysteine was the (sole?) cause for the increase in hydrogen production to a peak of 2.5 ml $min^{-1}$ $l^{-1}$ on day 7, which was associated with increased acetate and butyrate production as well as increased substrate conversion.

On days 8 and 9 hydrogen, carbon dioxide and butyrate production decreased again, the redox potential increased to –65mV, and acetate concentrations increased to 83.5 mmol $l^{-1}$. A second injection of L-cysteine to 0.1% on day 9 decreased the redox potential immediately to –320mV, similar to the potential of –330mV reached through the first injection. This time the drop in redox potential was associated with an instant drop in hydrogen production from 0.6 to 0.3 ml $min^{-1}$ $l^{-1}$, which then however was followed by an increase in hydrogen production to 0.9 ml $min^{-1}$ $l^{-1}$ on day 10, associated with a slight increase in butyrate concentration and a strong increase in acetate production to a concentration of 109.2 mmol $l^{-1}$ on day 10, when the experiment was ended due to time constraints.

## 3.3 *Discussion of experiments on starch*

### *3.3.1 Hydrogen yields*

In self generated gas atmosphere continuous hydrogen production was achieved at pH 4.5 and 35°C for 14 and 7 days (experiments St2 and St3) and at pH 5.2 and 30°C for 10 days (experiment St4), with average daily hydrogen yields of 1.3 mol per mol hexose converted for at least 3 to 5 days (period limited by data collection) in both environmental conditions.

When the culture was sparged with nitrogen gas, stable continuous hydrogen production was achieved for 18 days and hydrogen yields could be increased by 50% through sparging, from 1.3 mol hydrogen per mol hexose converted (experiments St3 and St4) to 1.9 mol hydrogen per mol hexose converted (experiment St7). Although sparging results in a significant dilution of the product gas, this may not be a significant problem in scale-up, as new gas separation systems utilizing polymeric membrane and active membrane technology can separate hydrogen from other gases such as nitrogen, methane and carbon dioxide at ambient temperatures and pressures (Teplyakov *et al.* 2002).

Doubling the organic loading rate by increasing the substrate concentration from 10 to 20 g $l^{-1}$ when sparging with nitrogen did not give rise to an increase in the hydrogen production rate, although over 80% of the 20g $l^{-1}$ starch was converted during continuous operation until day 7 of experiment St8 and throughout experiment St9 with exception of day 6. Comparatively low maximum daily average hydrogen yields of 0.8 mol per mol hexose converted were achieved on days 2 and 5 of experiment St8, and 1.2 mol per mol hexose converted on day 8 of experiment St9.

### *3.3.2 Start-up*

Altogether 1 batch experiment and 8 batch start-ups were reported. Table 3-2 shows that the lag phase of 9 hours to the beginning of gas production in the batch experiment St1 was the shortest lag phase observed in batch start-ups where the sludge inoculum was heated to >90°C (experiments St1 to St7).

***Table 3-2. Lag phase to gas production and product concentrations [mmol $l^{-1}$] at end of batch study/start of continuous operation***

| Exp. | Inoculum storage duration [days] | Lag phase [h] | Butyrate | Acetate | Ethanol | Lactate |
|---|---|---|---|---|---|---|
| St1 | 20 | 9 | 15.3 | 8.5 | 11.1 | n/d |
| St2 | 34 | n/d | 6.6 | 2.0 | n/d | n/d |
| St3 | 32 | 14 | 11.2 | 3.0 | 7.6 | n/d |
| St4 | 74 | 19 | 17.2 | 9.2 | 8.7 | n/d |
| St5 | 6 | 16 | n/d | n/d | n/d | n/d |
| St6 | 6 | 16 | 26.6 | 15.4 | 6.5 | n/d |
| St7 | 147 | 17 | 24.4 | 5.0 | 2.0 | n/d |
| St8 | 55 | 8 | 14.6 | 6.4 | 5.0 | 66.8 |
| St9 | 15 | 8 | 17.7 | 5.1 | 7.1 | 57.1 |

The storage duration of the inoculum was found to have no clear effect on the lag phase to gas production. This would be expected, as the heat treatment selects for spore formers, and spores are known to survive much longer than the durations required here (section 1.3.1.1). The lag phase was shortest in experiments St8 and St9, which differed from the other experiments in various points. Whilst the inoculum was heated to just over 90°C in experiments St1 to St7, it was heated to 110°C in experiments St8 and St9, which were carried out last of all experiments. This change was made to ensure that all vegetative cells would be killed in the heating process and thus non-spore formers such as propionibacteria and lactobacilli would be completely excluded. 110°C was assumed to be the highest temperature which would not also kill spores. Both temperatures, 90 and 110°C, should be sufficient to make the germination receptor more responsive to the presence of germinants (Johnstone 1994), since for *Clostridium botulinum* for example heating to 60°C for 15 min was shown to be sufficient (Plowman and Peck 2002). The more vigorous heating to 110°C may possibly have freed more germination inducing compounds from the sludge matrix. Also, the substrate concentration was doubled in experiments St8 and St9. This may provide any germination inducing compounds present in the substrate in twice the concentration compared to experiments St1 to St7. There was no difference in inoculum pre-treatment or substrate concentration between experiments St1 and experiments St2 to St7, therefore there are no obvious possible causes for the comparatively short lag phase in the batch experiment St1.

In all experiments hydrogen was present in the produced gas during batch operation, possibly with exception of experiments St5 and St6, where hydrogen was not determined at the beginning of continuous operation. Table 3-2 shows product concentrations at the end of gas production during the batch study St1 or at the

beginning of continuous operation in experiments St2 to St9. Although the beginning of continuous operation was to some extend arbitrary (after start of measurable gas production but limited by working hours), product concentrations at that point give an indication of the main products during batch start-up. In experiments St1 to St7 butyrate, acetate and ethanol were the only detected products. In experiments St8 and St9, the only experiments in which lactate and formate were determined, lactate was also detected. No propionate, acetone or butanol were detected in any of the experiments. Table 3-2 shows that butyrate was the main product at the end of batch operation in experiments St1 to St7. Butyrate production was accompanied by acetate and ethanol production in all experiments, with the ethanol concentration at the end of batch operation exceeding the acetate concentration in 3 out of 7 experiments (where both products were determined). Since ethanol production is not associated with hydrogen production but competes for carbohydrate substrate (equation 6), its suppression would be desirable. From Table 3-2 it can not be concluded that there is a clear difference in product distribution during batch start-up at pH 4.5 compared to pH 5.2, or in experiments with sparging compared to experiments with self generated gas atmosphere. In experiments St8 and St9 lactate was by far the main product. It is likely that lactate was also produced during the batch (start-up) in experiments St1 to St7, which may partly explain the large percentage of converted carbon not accounted for in the carbon balance for experiment St1. Like ethanol, lactate production is not associated with hydrogen production but competes for carbohydrate substrate (equation 6), and its suppression would be desirable.

### *3.3.3 Butyrate and acetate production during continuous operation*

As expected from the literature review, butyrate and acetate were the main fermentation products in the reactor effluent during times of continuous hydrogen production. Butyrate and acetate concentrations during periods of continuous operation with maximum hydrogen yields are summarised in Table 3-3. Table 3-3 shows that acetate and butyrate concentrations were highly variable, ranging from 3.5 to 27.2 mmol $l^{-1}$ and from 14.6 to 28.3 mmol $l^{-1}$ respectively. During all periods shown in Table 3-3 butyrate concentrations were equal to or larger than acetate concentrations, giving butyrate/acetate ratios in the range of 1.0 to 2.0 mol $mol^{-1}$.

***Table 3-3. Byrate and acetate concentrations during periods of continuous hydrogen production***

| Exp. | Days from start-up | Hydrogen yield [mol per mol hexose converted] | Acetate [mmol $l^{-1}$] | Butyrate [mmol $l^{-1}$] | Butyrate/acetate ratio [mol $mol^{-1}$] |
|---|---|---|---|---|---|
| St2 | 4 to 6 | 1.3 *) | 9.4 to 27.2 | 17.4 to 27.2 | 1.0 to 1.9 |
| St3 | 4 to 9 | 1.3 | 3.5 to 12.2 | 14.6 to 20.7 | 1.4 to 2.0 |
| St4 | 7 to 9 | 1.3 | 12.3 to 16.8 | 21.3 to 24.4 | 1.3 to 2.0 |
| St7 | 6 to 20 | 1.9 | 12.6 to 22.4 | 18.0 to 28.3 | 1.0 to 1.8 |

*) residual total hexose not determined, yield is therefore per mol hexose added

In the literature steady continuous hydrogen production is also generally associated with a butyrate/acetate ratio of greater than 1 (Table 3-4), but Fang and Liu (2002) report high hydrogen yields with a butyrate/acetate ratio of 0.6 mol $mol^{-1}$ (Table 3-4) from a bacilli rich mixed culture.

***Table 3-4. Butyrate/acetate ratio during continuous hydrogen production reported in the literature***

| Butyrate/ acetate ratio [mol $mol^{-1}$] | substrate | culture | Yield [mol $H_2$ $mol^{-1}$ hexose] | reference |
|---|---|---|---|---|
| 0.6 | glucose | mixed | 2.1 | (Fang and Liu 2002) |
| 1.7 | glucose | mixed | 1.4 | (Mizuno *et al.* 2000a) |
| 1.3 | glucose | *C. butyricum* | 2.2 | (Heyndrickx *et al.* 1990) |
| 1.3 | sucrose | mixed | 2.2 | (Liu and Fang 2002) |
| 2.6 | sucrose | mixed | 0.8 | (Chen and Lin 2003) |
| 1.3 | starch | mixed | 2.1 | (Lay 2000) |

It can be concluded that in the continuous hydrogen production described here and in publications listed in Table 3-4, there is theoretically plenty of scope to increase yields if the process could be biased towards acetate production as in equation 1.

In contrast to expectations based on equation 1, it was observed in the experiments in self generated gas atmosphere at 18 h HRT that periods of low or absent hydrogen production often coincided with very high acetate concentrations. In experiment St5 for example the acetate concentration ranged from 39.5 to 45.4 mmol $l^{-1}$ during days 6 to 9, the period of lowest to absent hydrogen production (Figures 3-5a and b). These acetate concentrations clearly exceeded those observed during periods of strong hydrogen production (Table 3-3) and, in combination with low butyrate concentrations, caused a low butyrate/acetate ratio of 0.2 to 0.3 mol $mol^{-1}$. Similarly, during days 13 to 16 of experiment St6, low daily average hydrogen yields of 0.5 to 0.7 mol per mol hexose converted were associated with high acetate concentrations of 34.6 to 40.0 mmol $l^{-1}$

(Figure 3-6b), which, in combination with low butyrate concentrations, gave a butyrate/acetate ratio of 0.4 to 0.5 mol $mol^{-1}$. Since the low or absent total gas production during days 6 to 9 of experiment St5 excludes the possibility of significant methane production, hydrogen consumption through homoacetogenesis is a likely explanation. As discussed in section 3.1.2.3 signs of homoacetogenesis were also observed during days 12 to 14 of experiment St2 at pH 4.5, therefore homoacetogenesis may have been present at pH 4.5 and pH 5.2 at 18 h HRT.

Shortening of the retention time to 12 hours in experiments St5 and St6 did not have a clear effect on homoacetogenesis. In experiment St5 the acetate concentration increased overall when the HRT was decreased from 18 to 12 hours (Figure 3-5b; acetate decreased from 32.2 to 23.9 mmol $l^{-1}$ followed by an increase to 40.8 mmol $l^{-1}$), but the hydrogen yield increased also, from 0.2 mol per mol hexose converted at the last day of operation at 18 h HRT to 0.5 to 0.9 mol per mol hexose converted at 12 h HRT. In experiment St6 on the other hand the acetate concentration decreased from 34.6 to 27.6 mmol $l^{-1}$ when the HRT was reduced from 18 to 12 hours (Figure 3-6b), whilst the daily average hydrogen yield did not change and ranged from 0.5 to 0.8 mol per mol hexose converted at 18 and 12 h HRT. The further decrease in HRT to 9 h on day 20 of experiment St5 caused a decrease in the acetate concentration from 40.8 to 29.5 mmol $l^{-1}$, but the average daily yields also decreased to 0.1 and 0.2 mol hydrogen per mol hexose converted. The change in acetate concentrations with change in hydraulic retention time can therefore not entirely be attributed to hydrogen production (equation 1) or hydrogen consumption (equation 10), but is most likely caused by change in intensity of both metabolisms. It is not clear from these results if shortening of the HRT discourages homoacetogenesis.

Since clostridia are amongst the homoacetogenic organisms (section 1.3.3), and some strains may even be able to switch from hydrogen production to hydrogen consumption (section 1.6.1), it may not be possible to exclude homoacetogenesis through change of pH, temperature or retention time. The most obvious method to discourage homoacetogenesis would therefore be reduction of the hydrogen partial pressure so as to make hydrogen consumption energetically unfavourable.

It was therefore proposed that sparging with nitrogen would discourage homoacetogenesis, which was investigated in experiments St7 to St9. In Experiment St7, where the highest hydrogen yields over the longest period were achieved, butyrate concentrations exceeded acetate concentrations until day 19. Overall the butyrate/acetate ratio decreased during continuous operation, but this did not affect the hydrogen yield. On day 8 a daily average hydrogen yield of 2.12 mol per mol hexose converted was associated with a butyrate/acetate ratio of 1.8 mol $mol^{-1}$ (butyrate and acetate concentrations of 26.3 and 15.0 mmol $l^{-1}$ respectively; Figure 3-7b). On day 20, the last day of the experiment, the same daily average yield was associated with a butyrate/acetate ratio of 1.0 mol $mol^{-1}$ (butyrate and acetate concentrations of 18.4 and 20.6 mmol $l^{-1}$ respectively). This indicates that in experiment St7 the decrease in the butyrate/acetate ratio was more likely due to a slight shift from hydrogen production in association with butyrate production (Equation 2) to hydrogen production in association with acetate production (Equation 1), rather than due to gradual development of homoacetogenesis.

In contrast to experiment St7, the acetate concentrations varied strongly in experiments St8 and St9 (also with sparging, but with increased substrate concentration of 20 g $l^{-1}$ compared to 10 g $l^{-1}$ in experiments St1 to St7). Also, the maxima in acetate concentration of 66 and 109 mmol $l^{-1}$ in experiments St8 and St9 respectively by far exceeded double the maximum acetate concentration of 22 mmol $l^{-1}$ encountered during stable hydrogen production in experiment St7. Butyrate concentrations on the other hand were only slightly affected by the doubling of the organic loading rate in experiments St8 and St9 and ranged from 10 to 40 mmol $l^{-1}$ at 20 g $l^{-1}$ substrate (experiments St8 and St9) compared to 5 to 30 mmol $l^{-1}$ at 10 g $l^{-1}$ substrate (experiment St7). It was considered whether the comparatively low hydrogen yields of 0.1 to 1.2 mol per mol hexose observed at the higher feed concentration of 20 g $l^{-1}$ in experiments St8 and St9 could be caused by product inhibition due to increased concentrations of undissociated butyric acid (section 1.7.6). At the operating pH of 5.5 approximately 11.4 mmol $l^{-1}$ of the maximum 40 mmol $l^{-1}$ butyrate observed in experiments St8 and St9 would be undissociated. This concentration exceeds the concentrations of 6.84 mmol $l^{-1}$, which has been shown by Chin *et al.* (2003) to decrease the total amount of hydrogen produced by a pure culture of *Clostridium acetobutylicum* by 30% (section 1.7.6). It is therefore possible that hydrogen production was affected by higher butyrate

concentrations, but as discussed in section 1.7.6, the critical undissociated butyric acid concentrations vary between studies. Zoetemeyer *et al.* (1982b) for example report 90 % glucose conversion and hydrogen yields of 1.43 mol per mol glucose at undissociated butyric acid concentrations of 16 mmol $l^{-1}$.

As observed in experiments with self generated gas atmosphere (for example experiments St2 and St5), increased acetate concentrations were not associated with an increase in hydrogen yields. In experiment St8 for example the daily average hydrogen yield was 0.7 mol per mol hexose converted on days 3 and 6, which was associated with a similar range of butyrate concentrations, 25.6 to 29.1 mmol $l^{-1}$ on day 3 and 25.3 to 29.9 mmol $l^{-1}$ on day 6, whilst the acetate concentrations increased from 13.5 to 15.4 mmol $l^{-1}$ on day 3 to 60.2 to 65.7 mmol $l^{-1}$ on day 6 (Figure 3-8b), resulting in a decrease of the butyrate/acetate ratio from 1.9 mol $mol^{-1}$ on day 3 to 0.4 mol $mol^{-1}$ on day 6.

Similarly, at the end of experiment St9 (days 9 and 10) acetate concentrations were extremely high (reaching 109 mmol $l^{-1}$), whilst daily average hydrogen yields were low with 0.3 and 0.2 mol per mol hexose converted. The low hydrogen yields observed in experiments St8 and St9 could therefore be due to development of homoacetogenesis, as was proposed for experiments St2, St5 and St6. Since methane was not determined in experiments St8 and St9, presence of methanogenesis is also a possible explanation for the low hydrogen yields in experiments St8 and St9. However, it can be assumed from observations in experiments without gas sparging, where methane was determined but never detected at pH 5.2 or 4.5, that homoacetogenesis is the more likely cause. This indicates either that lowering of the hydrogen partial pressure through sparging alone does not necessarily inhibit homoacetogenesis, or that sparging here did not sufficiently lower the hydrogen partial pressure.

Presence of homoacetogenesis was therefore indicated at pH 4.5 and 5.2, and 30, 32 and 35°C, with or without sparging, and was not clearly inhibited at 18, 15 and 12 h HRT.

### 3.3.4 *Other fermentation end-products*

Methane and solvent production were the main metabolisms expected to interfere with continuous hydrogen production. Methane production however was only detected at pH 6.0 (results not included due to technical problems), and acetone or butanol production was not detected at all. As discussed in 1.3.1.3. clostridia have been used for industrial solvent production, and it was expected that solvent production would be a major obstacle in continuous hydrogen production. Since accumulation of undissociated butyric acid was identified as the main trigger mechanism of solvent production (section 1.3.1.3), it was expected that solvent production would be particularly an issue at higher feed concentrations. However, even at 20 g $l^{-1}$ substrate concentration the total butyrate concentration did not exceed 40 mmol $l^{-1}$. Since for industrial solvent production substrate concentrations were 5 to 10% (section 1.3.1.3), substrate concentrations here might need to be significantly increased to encounter problems with solvent production. Of the maximum of 40 mmol $l^{-1}$ total butyrate observed in the experiments here, only 11.4 mmol $l^{-1}$ are undissociated at pH 5.2, which is significantly lower than for example the trigger concentrations of undissociated butyric acid of 17 to 22 mmol $l^{-1}$ reported by Jones and Woods (1986) for *Clostridium acetobutylicum*.

Apart from the desired butyrate/acetate metabolism and homoacetogenesis (as discussed in section 3.3.3), ethanol, propionate and lactate production were the only other metabolic pathways detected.

Ethanol was mainly produced during batch operation with concentrations up to 11 mmol $l^{-1}$ (experiment St1; Figure 3-1b). In experiments with 10 g $l^{-1}$ substrate ethanol concentrations decreased to below 2 mmol $l^{-1}$ during continuous operation at pH 4.5 and 5.2, and were not affected by shortening of the retention times in experiments St5 and St6. In experiments St8 and St9 with 20 g $l^{-1}$ substrate concentration ethanol concentrations ranged from 1 to 13 mmol $l^{-1}$ during continuous operation (Figure 3-8b). Since substrate concentrations are lower during continuous operation than batch operation, and ethanol concentrations were higher at higher feed concentrations during continuous operation, it is possible that ethanol production is advantaged by higher feed concentrations. Since production of ethanol, a reduced end product, yields less energy for the bacteria than acetate production, it was thought that clostridia would only switch

from acetate to ethanol production if NADH + $H^+$ could not be regenerated through production of molecular hydrogen. It was proposed that nitrogen sparging would reduce the hydrogen partial pressure, thus increase the proportion of NADH + $H^+$ regenerated through production of molecular hydrogen (section 1.6.1) and reduce ethanol production. However, since the highest ethanol concentration during continuous operation was observed in experiment St8 (13.2 mmol $l^{-1}$; Figure 3-8b) there was no clear indication of reduction in ethanol production by nitrogen sparging.

Propionate production on the other hand, which like ethanol is not associated with hydrogen production but can compete for carbohydrate substrate (equation 15), was only observed during continuous operation. In experiments St5 and St6 at pH 5.2, 35°C and 18 h HRT propionate production began on days 8 and 10 respectively, reaching a concentration of 8.5 and 9.5 mmol $l^{-1}$ on days 13 and 14 respectively (Figures 3-5b and 3-6b). High propionate concentrations of 36 mmol $l^{-1}$ were also observed in an experiment at pH 4.5, 30°C and 18 h HRT (the experiment was not described in detail due to technical problems), which showed that the theory of Ren *et al.* (1997), that propionic fermentation in continuous operation can be avoided by operation at pH 4.5, can not generally be applied. As the genus *Propionibacterium*, one microbial group which can convert carbohydrate to propionate and acetate, has been reported to be slow growing (section 1.3.4), it was thought that reduction of the HRT might discourage propionate production. In experiments St5 and St6 the propionate concentration decreased gradually, from 7.7 to 3.0 mmol $l^{-1}$ and from 9.5 to 6.8 mmol $l^{-1}$ respectively, when the retention time was decreased from 18 to 12 hours (Figures 3-5b and 3-6b). The further decrease in retention time to 9h in experiment St5 did not cause a further decrease in propionate concentrations, which stayed constant at 3.0 mmol $l^{-1}$. These results indicate that shortening of the HRT to 12 hours may disadvantage propionate producers but not cause their complete washout. It was thought that production of propionate, a reduced end product like ethanol, might be discouraged by nitrogen sparging. In experiments St7 and St8 with nitrogen sparging propionate production was not observed. However, in experiment St9 with nitrogen sparging propionate concentrations of up to 14 mmol $l^{-1}$ (Figure 3-10b) were observed during continuous operation, showing that sparging with nitrogen does not necessarily discourage propionate production.

As discussed in section 1.3.4 the main bacterial groups which convert carbohydrate to propionate are not spore formers. Since the inoculum was heat treated in all experiments with starch, these propionate producers should not be supplied with the inoculum. Since propionate production occurred gradually, and only during continuous operation, it is possible that the propionate producers are gradually introduced, for example with the feed or water. Alternatively, the propionate could have been produced from lactate, a metabolism of which some clostridia are capable (section 1.3.4). In experiments St1 to St7 lactate was not determined, therefore no conclusions can be drawn. In experiment St9, lactate was abundant (30 to 40 mmol $l^{-1}$) during times of propionate production and may thus have served as substrate. Since it is not clear which substrate was used for propionate production, propionate production should be discouraged, as it may compete with hydrogen production for carbohydrate substrate. Experiments St5 and St6 indicate that reduction of the HRT is a possible way to discourage propionate production.

Lactate and formate production were only determined in experiments St8 and St9. Whilst formate was not detected in significant quantities, lactate was discovered to be an important product, particularly during start-up. At the beginning of continuous operation lactate was the main product in both experiments, with maximum concentrations of 66.8 mmol $l^{-1}$ on day 1 of experiment St8 (Figure 3-8b) and 57.1 mmol $l^{-1}$ on day 1 of experiment St9 (Figure 3-10b). It is likely that lactate production was also present in experiments St1 to St7. Since the inoculum was heat-treated to 110°C in experiments St8 and St9, the lactate was most likely produced by spore-formers, possibly some clostridial species.

### *3.3.5 Redox potential*

The redox potential was only monitored in experiments St8 and St9. In both experiments the redox potential decreased from the start and reached a minimum of -425mV and -450 mV respectively at the beginning of continuous operation. This indicates that initially there were organisms present in the mixed culture which could actively lower the redox potential. These facultative anaerobic organisms were either present in the inoculum, in which case they must be spore formers, since the inoculum was heated to >110°C, or they were added with the substrate or water, in which case they could also be non-spore formers. Clostridia, which are with few exceptions obligate anaerobes (section 1.6.5), are not thought to be able to consume oxygen and should thus

not be able to regulate the redox potential. However, several facultative anaerobes are known to be able to convert carbohydrate to hydrogen (sections 1.3.1 and 1.3.2), and have been found in cultures derived from anaerobic sludge inoculum (section 1.5). The *Enterobacteriaceae,* which have been most intensively investigated for hydrogen production, are non-spore formers and should therefore not have survived the heat-treatment. Bacilli on the other hand are facultative anaerobes which do form spores and have been found dominant in mixed hydrogen producing cultures. Fang and Liu (2002) for example report that analysis of the microbial community in continuous experiments with a mixed culture on glucose (pH 5.5, 36°C, 6 h HRT) achieving hydrogen yields of 2.1 mol per mol hexose showed that the culture was dominated by bacilli. Similarly, Sung *et al.* (2002) report from continuous hydrogen production by heat-treated anaerobic digester sludge on sucrose, that after day 15 the culture was dominated by *Bacillus laveolaticus*, a spore forming facultative anaerobe. These observations suggest that bacilli supplied with the inoculum could be responsible for the initial decrease in redox potential.

During continuous operation the redox potential increased stepwise in both experiments, particularly in association with deterioration of hydrogen production. During days of relatively strong hydrogen production (days 1 to 6 in experiment St8 and days 1,2,7 and 8 in experiment St9) the redox potential was below –250 mV. On day 7 of experiment St8 the increase in redox potential followed the decrease in gas production, whilst on days 2 and 3 of experiment St9 the increase in redox potential preceded the decrease in gas production. It is therefore not clear if the change in redox potential was a cause or effect of the change in hydrogen production.

One possible explanation for the gradual increase in redox potential during continuous operation could be that the facultative anaerobic organisms responsible for the initial decrease in redox potential have a slower growth rate than obligate anaerobic clostridia and thus be out-competed once they had provided a suitably low environment for the obligate anaerobes. This could hypothetically lead to the evolving obligate anaerobe culture not being able to consume the oxygen added with feed and water during continuous operation, leading to sporulation and washout. However, this hypothesis would imply that in experiment St8, where the redox potential only increased significantly once hydrogen production had ceased, the failure of hydrogen production

either had other causes, or that the facultative anaerobes were responsible for the bulk of hydrogen production. It would also suggest that the oxygen consuming organisms at the beginning of the experiment were spore formers present in the inoculum rather than organisms supplied with the substrate, because if they were supplied with the substrate, they would be added continuously, and thus their number should not decrease.

In work with pure clostridial cultures addition of a reducing agent such as cysteine to the medium is common practice to produce a reducing environment for growth (Taguchi *et al.* 1995). Injection of L-cysteine in experiment St9 clearly decreased the redox potential, but it is not clear if this decrease in redox potential was the (sole) cause for the increase in hydrogen production observed during the 24 hours following the cysteine injection, because hydrogen production had already started to increase slightly before the cysteine was injected.

# 4 Hydrogen production from sugarbeet and refined sugar

The selection of operating parameters for experiments with sugarbeet and sucrose substrate was based on conclusions from experiments on starch described in section 3, and observations reported in the literature. pH 5.2 was chosen because it was suitable for hydrogen production from starch (see section 3). Fang and Liu (2001), Van Ginkel *et al.* (2001) and Sung *et al.* (2002) report successful hydrogen production from sucrose based substrates at the slightly higher pH of 5.5, but it was decided to test pH 5.2, as less alkali would be required for control of the lower operating pH, reducing the operating costs at potential scale-up. Similarly, the temperature was controlled at 32°C, because it was within the range (30 and 35°C) that allowed hydrogen production from starch (see section 3) and is within the range of operating temperatures at which hydrogen production from sucrose is reported in the literature, stretching from 26°C (Fang and Liu 2001) to 37°C (Sung *et al.* 2002).

The methodology for the experiments discussed here is given in section 2.7.2. Results from experiments Su8, Su9 and Su10 were published in Hussy *et al.* (2005).

## *4.1 Sugar content of sugarbeet*

Analysis of randomly selected chips of frozen beet showed that the peeled beet after storage at -20°C contained approximately 22 to 27% total solids and 180 to 210 g total sugar $kg^{-1}$ wet sugarbeet, which is higher than the average sucrose concentration of approximately 170 g $kg^{-1}$ for commercially refined British sugar beet (British Sugar 2005). With increasing storage time at -20°C the sugar content increased to 225 and 260 g total sugar $kg^{-1}$ wet sugarbeet (2 samples) after 12 months due to gradual dehydration of the frozen beet.

After separation of the water extract and water-extracted pulp for continuous feeding in experiments Su9 and Su10 (for details see section 2.3.2), the water extract was found to contain 38 to 60 g total sugar $l^{-1}$ and the residual pulp 5.5 to 7.6 g total sugar $kg^{-1}$ wet weight (Table 4-1).

*Table 4-1. Sugar content of water extract and pulp in each extraction batch.*

| Extraction batch | Used in experiment | During days from startup | Wet weight of sugarbeet extracted [g ] | Sugarbeet water extract obtained [ml] | Sugar content of sugarbeet water extract [g $l^{-1}$] | Extracted wet pulp obtained [g] | Sugar content of extracted wet pulp [g $kg^{-1}$] |
|---|---|---|---|---|---|---|---|
| 1 | Su8 | 34-38<br>41-43 | 1600 | 5300 | 52.2 | 500 | 6.1 |
| 2 | Su8<br>Su9 | 44-45<br>11-16 | 1600 | 5620 | 57.8 | 370 | 5.5 |
| 3 | Su9 | 16-18<br>25-30 | 1936 | 6740 | 59.4 | 560 | 5.5 |
| 4 | Su9 | 30-32 | 1722 | 6250 | 38.0 | 440 | 7.6 |

From Table 4-1 can be calculated that in all extraction batches over 98.5 % of total sugar was extracted into water. For extraction batch 1 for example 5300 ml sugarbeet water extract containing 52.2 g $l^{-1}$ total sugar was obtained, therefore a total of 277 g total sugar was extracted into water. 500 g wet pulp remained, containing 6.1 g $kg^{-1}$ total sugar, which means that a total of 3 g total sugar remained in the pulp. 99% of total sugar was therefore extracted into water for extraction batch 1. In experiment Su9 the daily total sugar supplied with the pulp during days 34 to 38 and 41 to 45 (see Table 2-5) was approximately 1% of the total sugar supplied with the water extract.

### *4.2 Batch experiments*

In 4 of the 5 batch experiments (Su1 to Su4) with heat treated anaerobic digester sludge and whole sugarbeet pulp hydrogen production started after a lag phase of 19 to 21 hours and continued for up to 12 hours (Figure 4-1). In experiment Su5 gas production started after a lag phase of just 9 hours and continued for 17 hours. There is no obvious reason for this earlier start of gas production in experiment Su5.

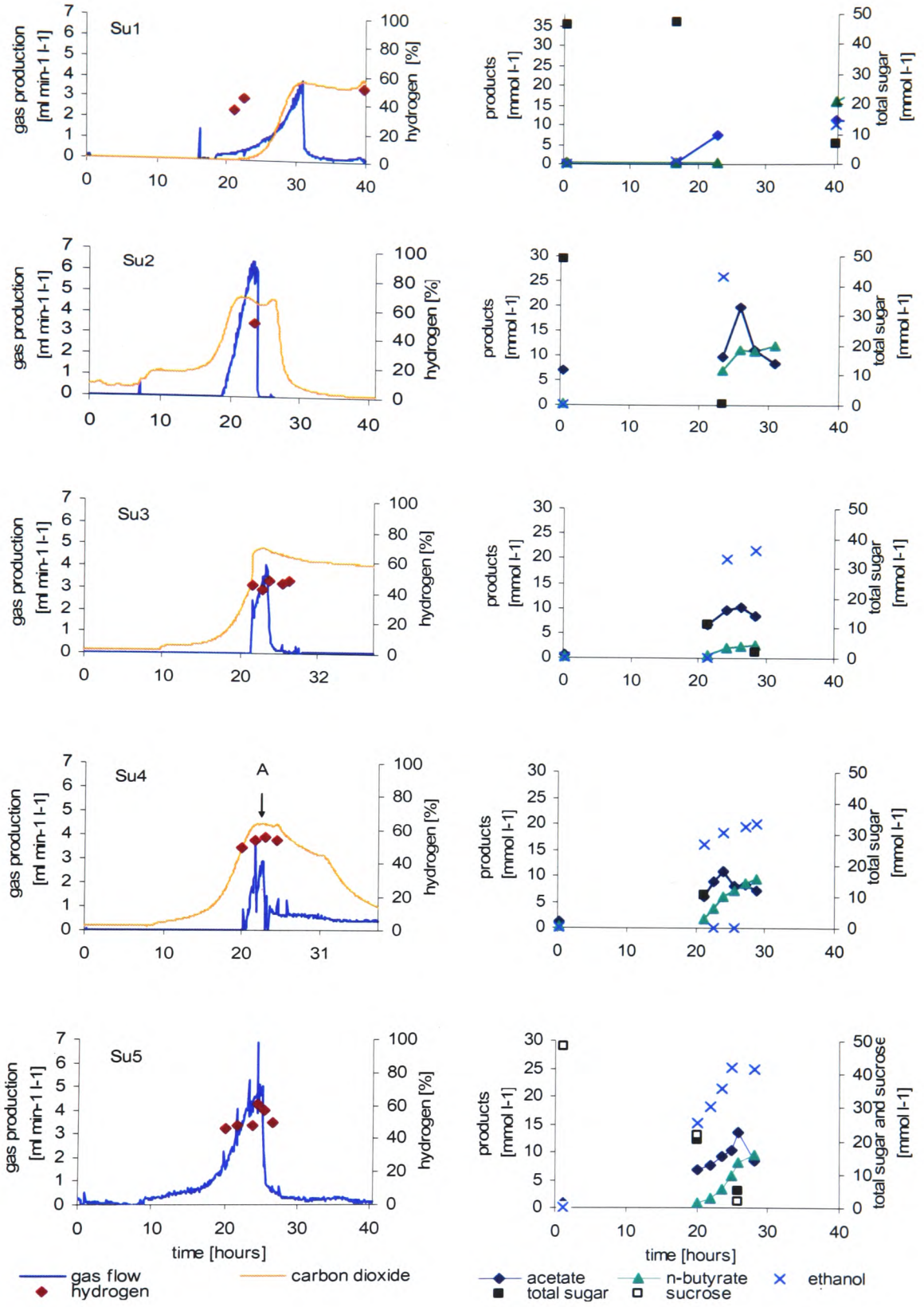



***Figure 4-1. Batch experiments Su1 to Su5 with pulped sugarbeet (100g wet weight per reactor volume) and heat-treated inoculum. Arrow A: gas meter changed from LFM 300 to ADM 2000. Left: gas production [ml $min^{-1}$ $l^{-1}$] and hydrogen content [%]. Right: products and residual total sugar [mmol $l^{-1}$], residual sucrose [mmol $l^{-1}$] in exp. Su5 only.***

The total gas production during these experiments ranged from 0.3 l gas $l^{-1}$ reactor (experiment Su3) to 1.7 l gas $l^{-1}$ reactor (experiment Su5), containing 40 to 60% hydrogen. Hydrogen yields in experiments Su1 to Su4 could only be estimated. Calculations were limited by problems with total sugar determination and off-line data. In experiment Su1 the hydrogen content of the produced gas was not determined during the peak of gas production. In experiment Su2 the hydrogen content was only determined once. In experiments Su3 and Su4 the sugar concentration at the start of the experiments was not reliably determined. Based on the assumptions that the hydrogen content of the produced gas in experiment Su1 and Su2 was 50% throughout and the initial total sugar concentration in experiment Su3 and Su4 was 48 mmol $l^{-1}$ (average of values from Su1, Su2 and Su5), hydrogen yields for experiments Su1 to Su4 were less than 1 mol per mol hexose converted. The hydrogen yield for experiment Su5 was 1.1 mol per mol hexose converted.

Unlike in experiments with starch (section 3), where butyrate was the main product during batch start-up, here more acetate than butyrate was produced during the main period of gas production (Figure 4-1; no data for experiment Su1 at that point). Also, whilst in batch start-ups on starch ethanol concentrations were generally below 12 mmol $l^{-1}$ and did not exceed butyrate concentrations, ethanol was the main determined product in experiments Su1 to Su5 with a concentration of 19 to 26 mmol $l^{-1}$ (Figure 4-1) at 23 to 25 hours after start-up. Ethanol was also found as a major product in batch experiments by Oh *et al.* (2004) on 13 mmol $l^{-1}$ glucose inoculated with anaerobic sludge inoculum with and without heat treatment. It appears therefore that strong ethanol production in batch fermentation is not population specific. Since ethanol production is not associated with hydrogen production, but competes with butyrate/acetate production for carbohydrate substrate (section 1.3.1.4.), it may partly be the cause of the relatively low hydrogen yield. To test whether ethanol producers were imported into the reactor with the sugarbeet, the sugarbeet pulp was taken to >90°C for 15 minutes in experiment Su4 and autoclaved in experiment Su5. Heat treatment or autoclaving of the substrate did not inhibit ethanol production, suggesting that the ethanol producers are most likely spore-formers. No propionate, acetone or butanol production was detected. Lactate and formate concentrations were not determined.

## *4.3 Continuous experiments*

### *4.3.1 Experiments Su6 and Su7*

From observations in experiments St3 to St6 on starch (Figures 3-3b, 3-4b, 3-5b and 3-6b), where ethanol production decreased during continuous operation, it was concluded that butyrate production may be favoured over ethanol production at lower substrate concentrations. Since continuous operation will result in a lower substrate concentration in the reactor than batch operation, lower ethanol and higher butyrate concentrations and a greater hydrogen yield may be obtained in continuous operation. Refined sugar was used as substrate to test this hypothesis by proceeding to continuous operation at 12 h HRT after batch start-up in experiments Su6 and Su7. As in experiments Su1 to Su5, the inoculum was heat treated and whole sugarbeet pulp was used for batch start-up, however refined sugar at 10 g $l^{-1}$ was fed during continuous operation.

In experiment Su6 continuous feeding with refined sugar commenced 24 hours after start-up, when the hydrogen content of the produced gas was 49% (Figure 4-2a).

As observed in the batch experiments Su1 to Su5, ethanol was the main product during batch start-up, reaching a concentration of 14.5 mmol $l^{-1}$ at hour 24 (Figure 4-2b) compared to butyrate and acetate concentrations of only 2.0 and 6.9 mmol $l^{-1}$ respectively. As expected, ethanol production decreased during continuous operation, to 4.6 mmol $l^{-1}$ on day 3, whilst butyrate production increased to 30.4 mmol $l^{-1}$. The acetate concentration changed little with continuous operation, and no acetone, butanol or propionate was produced during the entire experiment.

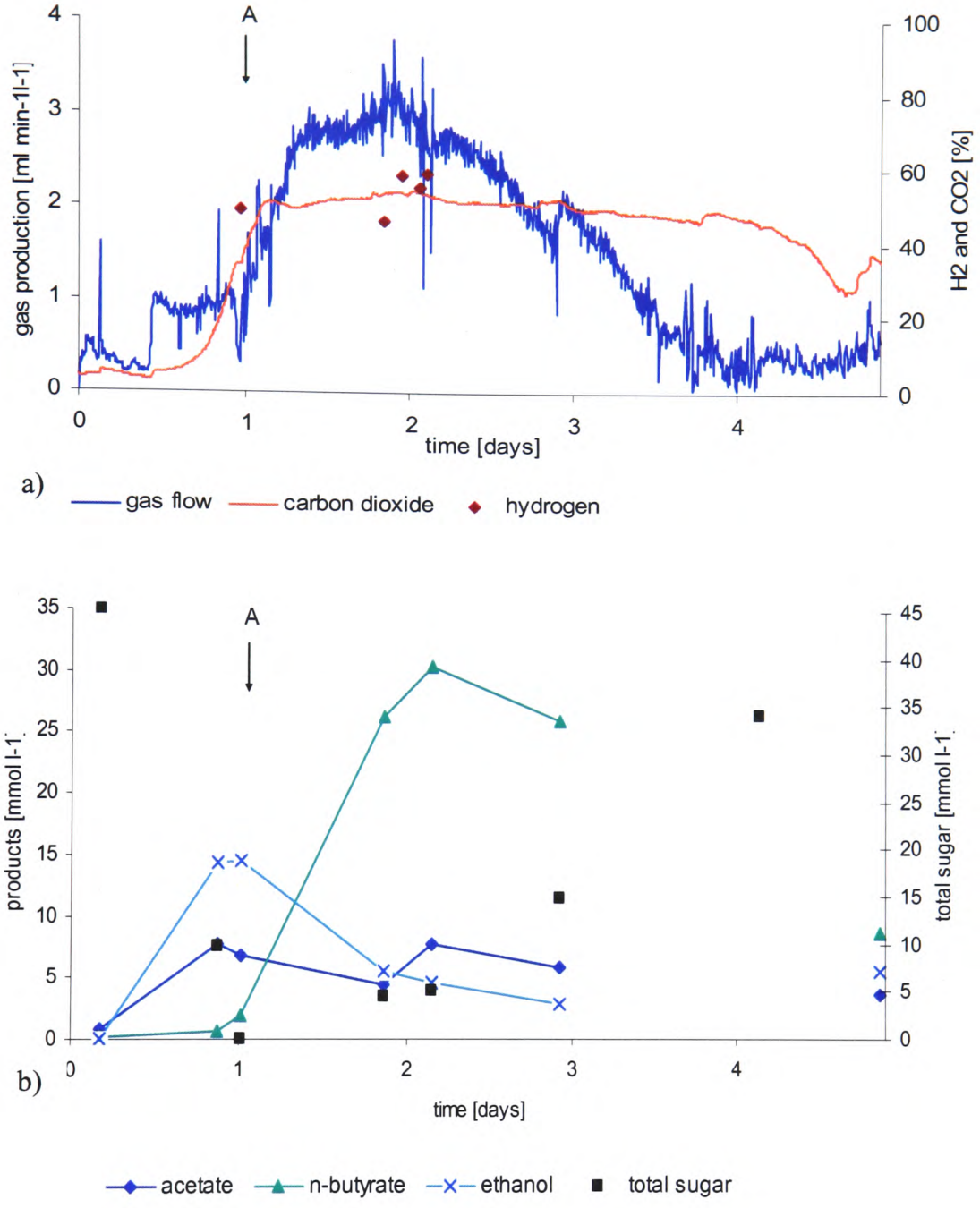



*Figure 4-2. Experiment Su6. Batch start-up on pulped sugarbeet and continuous operation on refined sugar at 12 h HRT without sparging. Arrow A: continuous operation started. a) gas production [ml ml$^{-1}$ l$^{-1}$] and composition [%] b) products and residual total sugar [mmol l$^{-1}$].*

During day 2 gas production increased from 1 ml min$^{-1}$ l$^{-1}$ (Figure 4-2a) to around 3 ml min$^{-1}$ l$^{-1}$, containing 50% hydrogen. During the peak in gas production (hours 36 to 48) the average hydrogen yield was 1.0 mol per mol hexose converted. However, on days 3 and 4 the gas production decreased steadily, to < 0.5 ml min$^{-1}$ l$^{-1}$ on day 5. This was

accompanied by a decrease in butyrate and acetate concentrations and a rise in total sugar concentration to 34 mmol $l^{-1}$ on day 5, suggesting washout of the whole culture.

In experiment Su7 very similar observations were made: ethanol was also the main product during start-up, reaching a concentration of 15.5 mmol $l^{-1}$ at hour 27, compared to butyrate and acetate concentrations of only 0.5 and 5.1 mmol $l^{-1}$ respectively (Figure 4-3b).

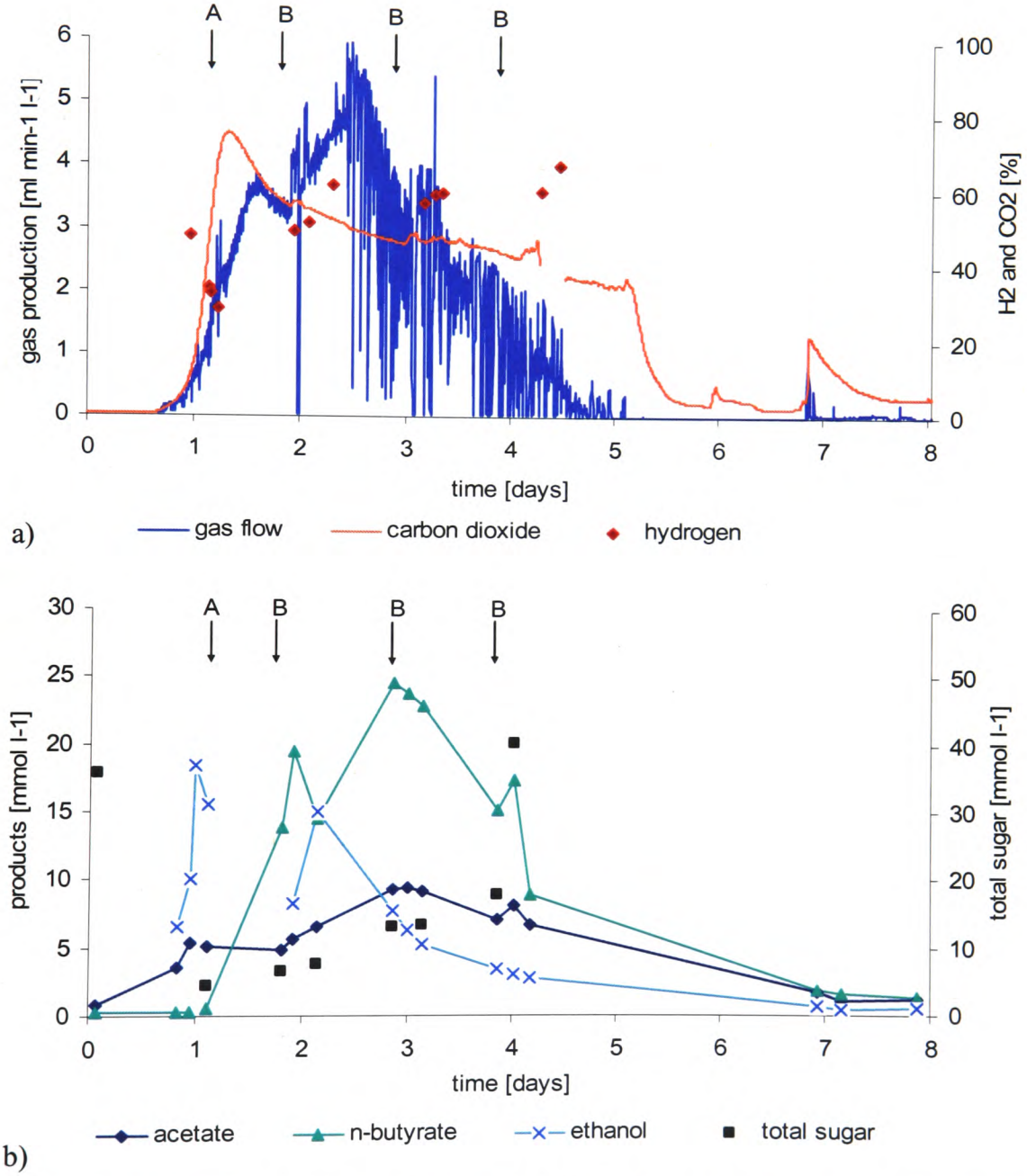



*Figure 4-3. Experiment Su7. Batch start-up on pulped sugarbeet with heat-treated inoculum and continuous operation on refined sugar at 12h HRT without sparging. Arrow A: continuous operation started. Arrow B: overflow pipe cleared. a) gas production [ml $min^{-1}$ $l^{-1}$] and composition [%] b) products and residual total sugar [mmol $l^{-1}$].*

Continuous operation was started at hour 27. As in experiment Su6 the ethanol concentration decreased with start of continuous operation, whilst butyrate concentrations increased. Here, the decrease in ethanol concentration was interrupted by a short period of strong ethanol production on day 2, which followed disturbances caused by blocking of the overflow pipe with sugarbeet pulp. By day 3 the ethanol concentration had decreased to 6.3 mmol $l^{-1}$, whilst the butyrate concentration had increased to 23.7 mmol $l^{-1}$.

The gas production increased with continuous operation, to a maximum of 5.5 ml $min^{-1}$ $l^{-1}$ on day 3, giving an average hydrogen yield of 1.2 mol per mol hexose converted during days 2 and 3. However, as in experiment Su6, gas production started to decrease on day 3, until here it ceased on day 5. As in experiment Su6, this was accompanied by decrease in butyrate, acetate and ethanol concentrations and increase in residual total sugar concentration, to 40 mmol $l^{-1}$ on day 4, indicating washout.

Despite the observed change of the dominant metabolism from ethanol to butyrate producing at the beginning of continuous operation in experiments Su6 and Su7, the hydrogen producing culture could not be sustained for more than 2 days of continuous feeding. There was no obvious explanation for the apparent washout from the available data. Apart from the regular blocking of the overflow pipe in experiment Su7 no technical problems were encountered, and propionate, acetone and butanol were not detected in either of the two experiments. In search of an explanation for these washouts the redox probe used in experiments St8 and St9 on starch, which were carried out after experiments on sugarbeet/refined sugar, was purchased. It was thought possible that the washout in experiments Su6 and Su7 was partly caused by dissolved oxygen entering with the feed (section 3.3.5). However, since dissolved oxygen would equally have been added in experiments St2 and St4 to St7 on starch, where washout was not observed, addition of dissolved oxygen with substrate and water alone could not have been responsible for the observed washout in experiments Su6 and Su7.

### *4.3.2 Experiment Su8*

Yokoi *et al.* (1998a) showed that addition of the facultative anaerobe *Enterobacter aerogenes* to a pure culture of *Clostridium butyricum* growing on starch not only enabled *Clostridium butyricum*, which otherwise required a reducing agent for growth, to convert the starch to hydrogen without reducing agent, but also achieved high hydrogen yields of 2 mol per mol glucose. On the basis of this information and considerations about the causes of washout in experiments Su6 and Su7, the sludge inoculum in experiments Su8 was not heat treated, and thus the non-spore forming anaerobes not selected against. This was thought to increase the number and variety of facultative anaerobes present in the inoculum.

Figure 4-4a shows that in experiment Su8, for which sludge inoculum had been stored for 17 weeks, hydrogen production started after a lag phase of 60 hours. Unlike in experiments St8 and St9 (with heat-treated inoculum on starch), where the redox potential started to decrease immediately, here it did not decrease during the first 20 hours, and only started to drop rapidly after 43 hours. However, similar to experiment St8, the redox potential had decreased to -140 mV by the time hydrogen production started at hour 60.

Unlike in experiments Su1 to Su7, butyrate was the main product (Figure 4-4b) during batch start-up, reaching a concentration of 11.8 mmol $l^{-1}$ on day 3, compared to 8.1 mmol $l^{-1}$acetate and just 1.2 mmol $l^{-1}$ ethanol. From the product distribution here it was thought that ethanol production from sucrose may be advantaged by heat treatment of the inoculum.

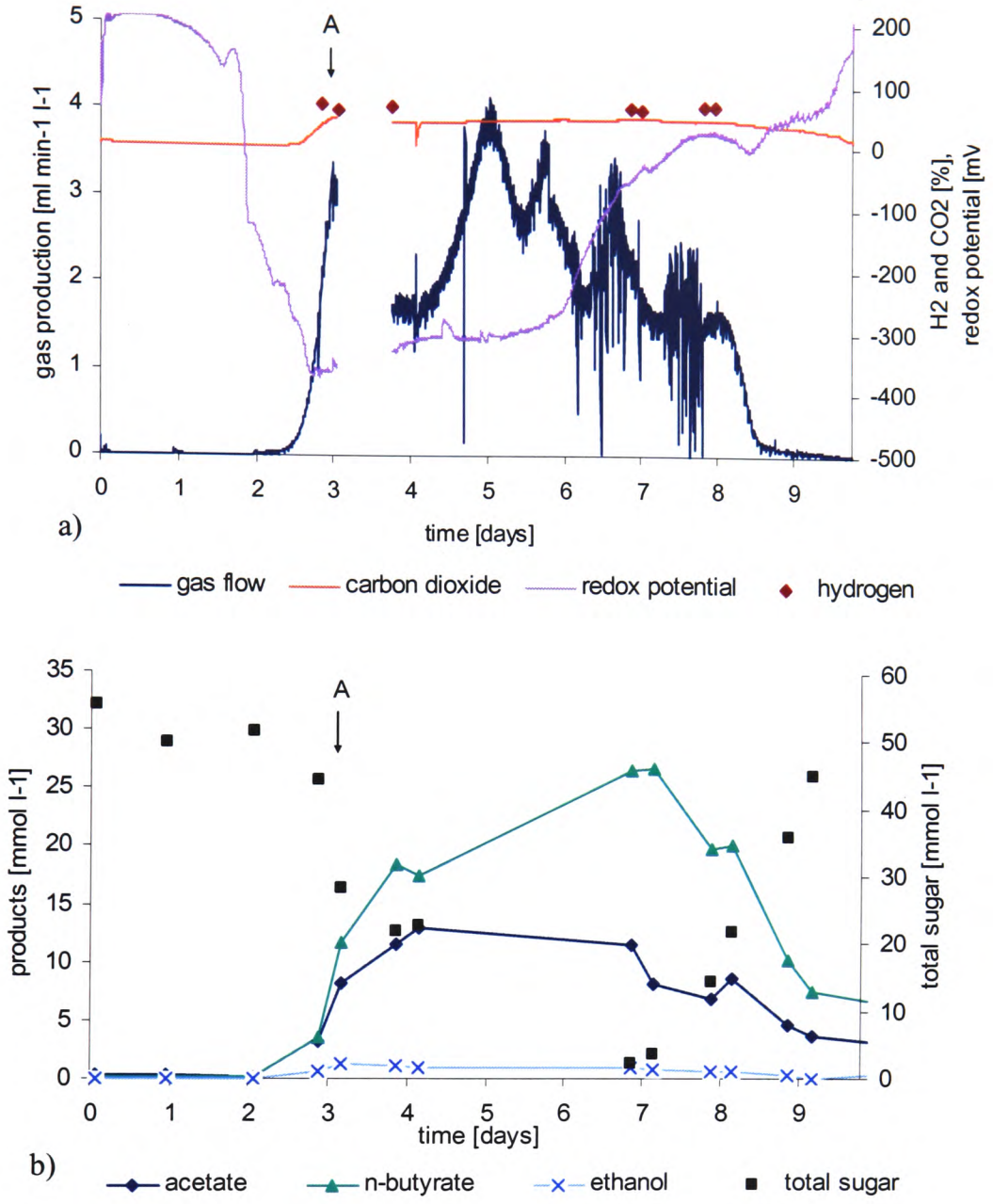



***Figure 4-4. Experiment Su8. Batch start-up and continuous operation on refined sugar without heat-treatment of inoculum at 15 h HRT without sparging. Arrow A: continuous operation started. a) gas production [ml min$^{-1}$ l$^{-1}$] and composition [%], redox potential [mV] b) products and residual total sugar [mmol l$^{-1}$].***

Continuous operation commenced at hour 74 (start of day 4) by feeding of refined sugar. During days 4 to 7 the gas production varied from 2 to 4 ml min$^{-1}$ l$^{-1}$ in oscillations (Figure 4-4a). Butyrate continued to be the main product, with a maximum concentration of 26.7 mmol l$^{-1}$ detected on day 7, compared to a maximum acetate concentration of only 13.0 mmol l$^{-1}$ on day 4. Throughout continuous operation the redox potential increased gradually (Figure 4-4a) from –350 mV on day 4 to –280 mV

on day 6, but steeply thereafter, reaching +30 mV on day 8 and +160 mV on day 10. From day 7 clear signs of washout appeared. During days 7 to 9 the residual total sugar concentration increased from 2.2 to 44.9 mmol $l^{-1}$, whilst butyrate and acetate concentrations decreased from 26.7 and 8.2 mmol $l^{-1}$ to 6.7 and 3.2 mmol $l^{-1}$ respectively (Figure 4-4b). The gas production decreased gradually and ceased on day 9 (Figure 4-4a).

As in experiment St9 on starch it appears that the onset of increase in redox potential preceded signs of deterioration of the hydrogen producing culture: the strong increase in redox potential from day 6 preceded the decrease of total sugar conversion by one day, suggesting that here the redox potential could possibly be used as an early indicator for deterioration of the hydrogen producing culture.

As in experiments Su6 and Su7 there were no particular reasons for the washout, as there were no technical problems encountered or changes in the dominant culture metabolism detected. Propionic acid, acetone and butanol concentrations were < 0.7 mmol $l^{-1}$ and the butyrate/acetate and hydrogen producing metabolism (as in equations 1 and 2) was dominant throughout. This leaves the increase in redox potential, which here clearly preceded the washout, as a possible cause for the washout, as hypothesized in sections 3.3.5 and 4.3.1. Cohen *et al.* (1984) report butyrate/acetate and hydrogen producing metabolism to be associated with a redox potential of around –300 mV for a mixed culture, which is in accordance with the redox potential during days 3 to 6 of experiment Su8, but means that after day 6 the redox potential was possibly too high for hydrogen producers.

### *4.3.3 Experiment Su9*

It was thought that the long lag phase to gas production during start-up and the increase in redox potential during continuous operation in experiment Su8 could be explained by the fact that the bulk of vegetative cells of facultative anaerobes in the sludge inoculum might not have survived the long storage period of 17 weeks. Therefore fresher inoculum was used in experiment Su9. Additionally the reactor was sparged with nitrogen during start-up and continuous operation until a stable hydrogen producing culture was developed. Although (partial) washout and an increase in redox potential

during continous operation were also observed in experiments St8 and St9 on starch with sparging, it was expected that sparging would to some extent reduce oxygen as well as hydrogen concentrations in the reactor.

Sparging was used throughout batch start-up and continuous operation on refined sugar until day 17, when sparging was stopped so that hydrogen yields for refined sugar in sparging and non-sparging conditions could be compared. From days 34 to 38 and 41 to 45 sugarbeet water extract and water extracted pulp were used as substrate so that hydrogen yields in non-sparging conditions could be compared with those for refined sugar. A mass balance was calculated for 3 periods of experiment Su9, as shown in Table 4-2.

***Table 4-2. Mass balance [mg C $l^{-1}$] for 4 periods of experiments Su9 and Su10***

| Experiment | Su9 | Su9 | Su9 | Su10 |
|---|---|---|---|---|
| Days | 13.0 to 17.0 | 27.5 to 31.5 | 36.0 to 38.5 | 28.0 to 32.0 |
| TOC of liquid phase | 3669 | 3288 | 3383 | 2637 |
| VSS * | 1185 | 891 | | |
| TOC filtered | 2519 | 2406 | 1967 | 2028 |
| Acetate | 570 | 555 | 665 | 519 |
| Propionate | 26 | 29 | 26 | 5 |
| i-Butyrate | 17 | 30 | 27 | 27 |
| n-Butyrate | 1634 | 1457 | 983 | 1077 |
| i-Valerate | 5 | 6 | 6 | 11 |
| n-Valerate | 0 | 0 | 0 | 0 |
| Acetone | 0 | 0 | 0 | 0 |
| Ethanol | 42 | 27 | 89 | 231 |
| Butanol | 17 | 9 | 9 | 42 |
| Formate | 3 | 11 | 5 | 0 |
| Lactate | 0 | 0 | 0 | 2 |
| Residual sugar | 176 | 131 | 129 | 129 |
| Σ filtered carbon in liquid phase | 2486 | 2250 | 1935 | 2042 |
| % filtered TOC of liquid phase accounted for | 98.7 | 93.5 | 98.4 | 100.7 |
| Σ total carbon in liquid phase (includes VSS) | 3671 | 3141 | | |
| % total TOC of liquid phase accounted for | 100.1 | 95.6 | | |

* based on assumption that biomass contains 45.5 % carbon (Cohen *et al.* 1984)

#### 4.3.3.1 Operation on refined sugar with sparging

Figure shows that hydrogen and carbon dioxide production started within less than 16 h.

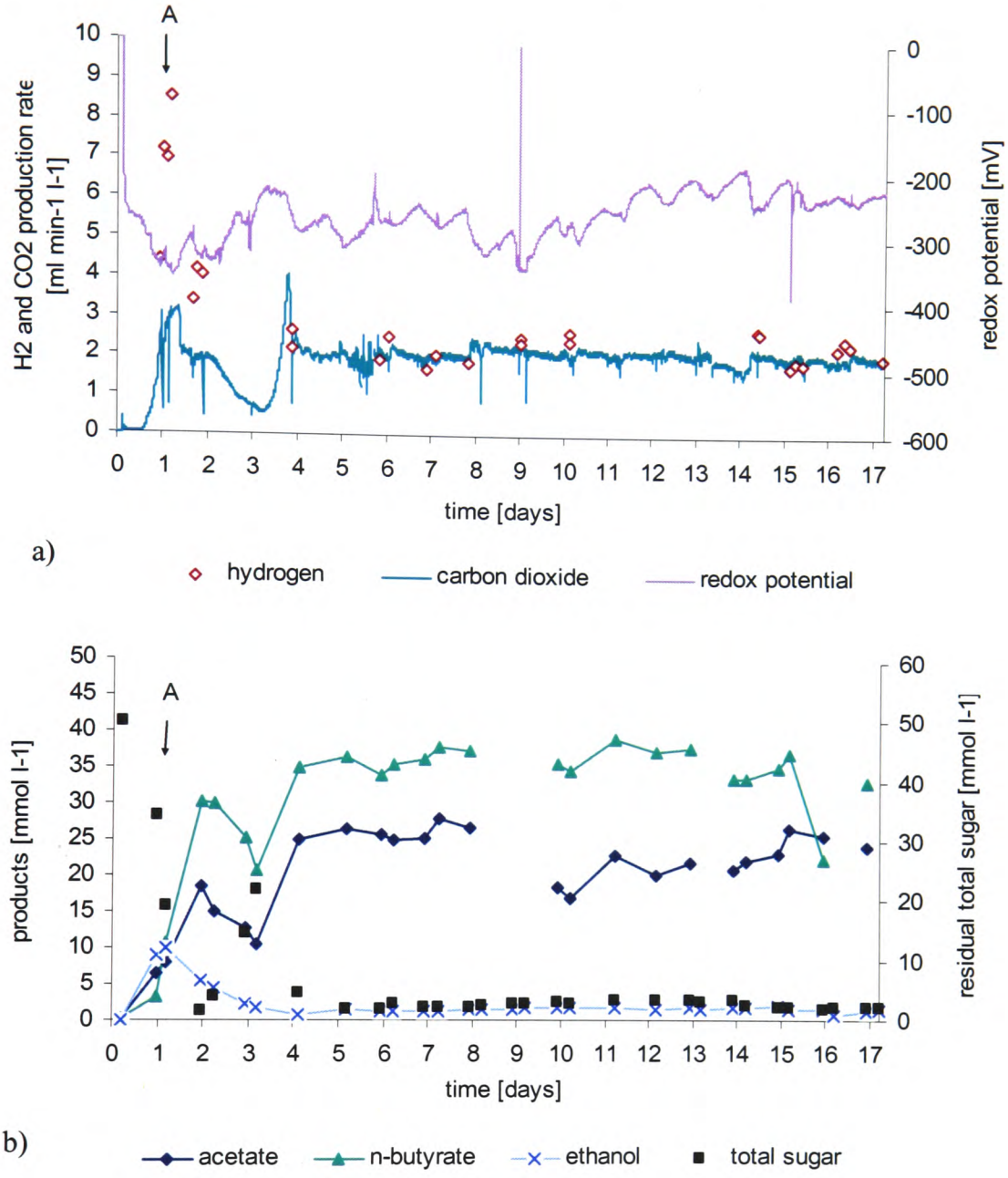



***Figure 4-5. Experiment Su9, days 1 to 17. Batch start-up and continuous operation at 15 h HRT on refined sugar without heat-treatment of inoculum but with sparging. Arrow A: continuous operation started a) hydrogen and carbon dioxide production [ml $min^{-1}$ $l^{-1}$] and redox potential [mV] b) products and residual total sugar [mmol $l^{-1}$].***

This lag phase was considerably shorter than that of 60 hours in experiment Su8 (Figure 4-4a) and approached the lag phase of 14 hours reported by Sung *et al.* (2002) from heat-treated sludge inoculum on sucrose at pH 5.5. The shortening of the lag phase

compared to experiment Su8 may be due to sparging and/or the use of fresher inoculum. The redox potential had decreased to –260 mV before gas production started, and decreased further to –330 mV (as in experiment Su8) by the time continuous operation commenced (Figure 4-5a). The decrease in redox potential to –260 mV happened within 6 hours in experiment Su9 compared to 43 hours in experiment Su8.

Figure 4-5a shows that in experiment Su9 hydrogen production increased to over 7 ml $min^{-1}$ $l^{-1}$ within the first 24 hours, giving with carbon dioxide a gas production of over 10 ml $min^{-1}$ $l^{-1}$, compared to 3 ml $min^{-1}$ $l^{-1}$ at the start of continuous operation (day 3) of experiment Su8. As in experiments Su1 to Su7 ethanol was the main product after 24 hours with a concentration of 8.9 mmol $l^{-1}$, when continuous operation was started (Figure 4-5b), showing that ethanol production from sucrose in batch mode does not depend on heat treatment. Butyrate and acetate concentrations after 24 hours were 3.2 and 6.6 mmol $l^{-1}$. The similarity of product distribution here and in experiments Su1 to Su7 shows that product distribution during batch start-up was not influenced by nitrogen sparging.

With the start of continuous operation butyrate production became dominant, reaching a concentration of 30.4 mmol $l^{-1}$ on day 2 (Figure 4-5b). Acetate concentration increased to 18.5 mmol $l^{-1}$ on day 2, whilst ethanol concentration decreased gradually, to < 1.7 mmol $l^{-1}$ on day 3, where it remained during continuous operation on refined sugar. After day 2 (after 2 retention times) signs of washout appeared as observed in experiment Su8, with butyrate and acetate concentrations decreasing to a minimum of 20.7 and 10.4 mmol $l^{-1}$ respectively (Figure 4-5b) on day 3, coinciding with an increase in residual total sugar concentration from 1.7 to 21.7 mmol $l^{-1}$ (Figure 4-5b), an increase in redox potential from –330 mV on day 2 to –230 mV, and a decrease in carbon dioxide production from 2 to 0.5 ml $min^{-1}$ $l^{-1}$ (Figure 4-5a). Unlike on day 8 of experiment Su8 however, washout was reversed after day 3.

During days 5 to 17 the redox potential, which had increased to –230 mV during the washout on day 3, varied strongly from –340 mV to –200 mV, with no clear relation to hydrogen production. Hydrogen and acid production were steady with an average hydrogen yield of 1.9 ± 0.2 mol per mol hexose converted. Butyrate was the main product with a concentration of 35.2 ± 3.8 mmol $l^{-1}$ compared to an average acetate

concentration of 23.6 ± 0.3 mmol $l^{-1}$. No acetone or lactate was produced, and ethanol, propionate and formate concentrations remained below 2 mmol $l^{-1}$. The VSS concentration, which was used here as a measure for bacterial biomass (since the feed was not particulate), was determined for days 10 to 17, and was found to decrease steadily from 5.2 g $l^{-1}$ on day 10 to 2.1 g $l^{-1}$ on day 17, contrasting with the steady product concentrations. Over 95% of substrate was consumed. The mass balance for days 13.0 to 17.0 (Table 4-2) shows that 100 % of the TOC in the liquid phase of the reactor was accounted for, confirming that there were no other significant fermentation products.

#### 4.3.3.2 Operation on refined sugar without sparging

On day 17 of experiment Su9 the sparging gas was turned off to investigate if this would cause a decrease in hydrogen yield or even washout. Data for the period without sparging are shown in Figure 4-6.

Hydrogen production decreased from 2.3 ml $min^{-1}$ $l^{-1}$ on day 17 (total exiting gas flow of 25.5 ml $min^{-1}$ $l^{-1}$, containing 9% hydrogen) to 1.1 ml $min^{-1}$ $l^{-1}$ on day 21 (gas production of 2.0 ml $min^{-1}$ $l^{-1}$, containing 55% hydrogen), but varied little during days 21 to 32 (Figure 4-6a).The average daily gas production during days 21 to 32 was 2.1 ± 0.4 ml $min^{-1}$ $l^{-1}$ with a hydrogen content of 54 ± 2 %. This gives an average daily yield of 1.0 ± 0.1 compared to 1.9 ± 0.2 mol hydrogen per mol hexose converted during days 5 to 17 with nitrogen sparging. During days 21 to 24 the non-gaseous product concentrations were very similar to those during nitrogen sparging: the average butyrate and acetate concentrations were 35.6 ± 0.9 mmol $l^{-1}$ and 26.3 ± 1.1 mmol $l^{-1}$ respectively, and > 95% of substrate was consumed (Figure 4-6b).

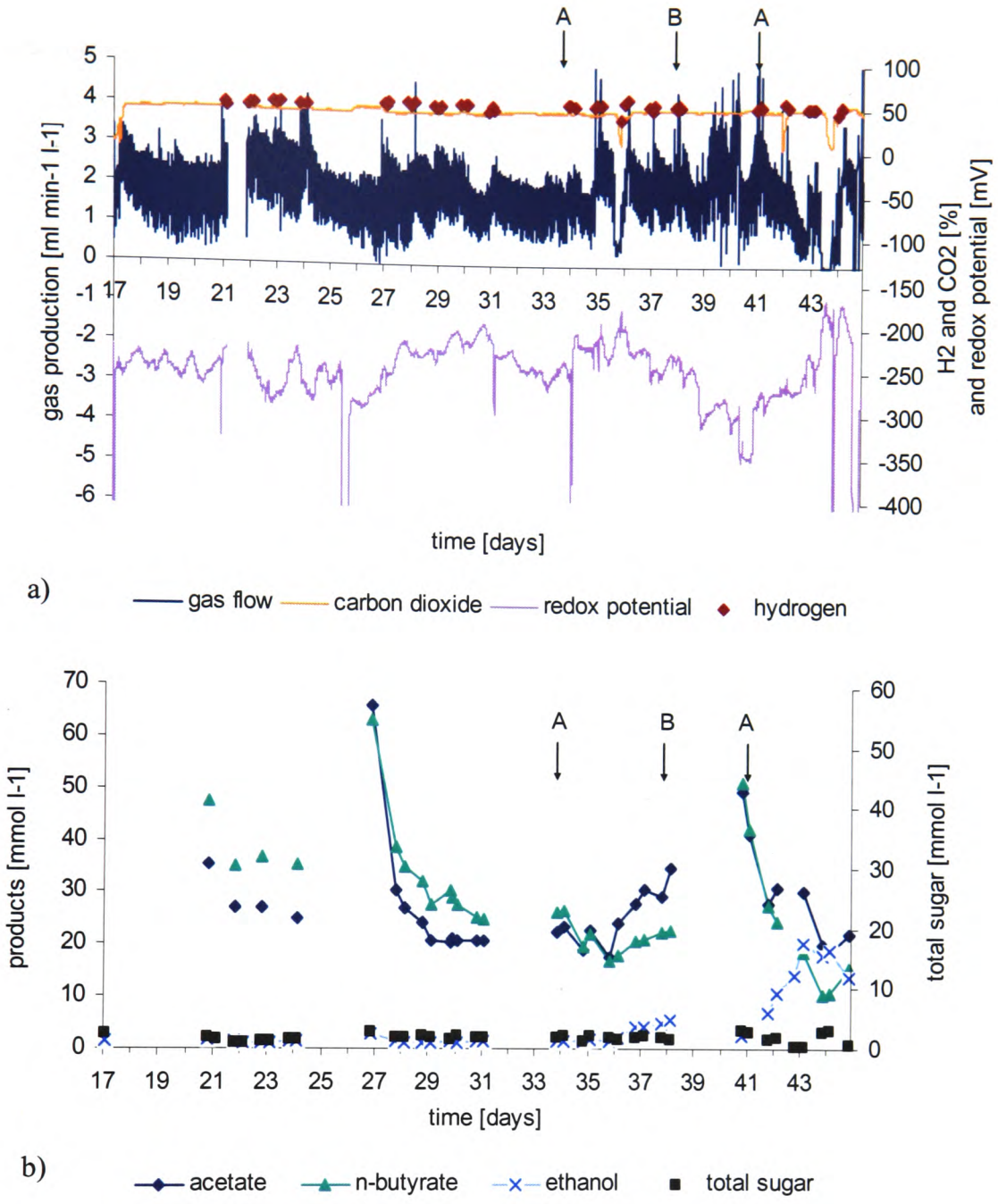



***Figure 4-6. Experiment Su9, days 17 to 45. Continuous operation on refined sugar (Days 17 – 34 and 38-41) or sugarbeet water extract (Days 34-38 and 41-45) without heat-treatment of inoculum and without sparging. Arrow A: operation on sugarbeet water extract started. Arrow B: operation on refined sugar recommenced. a) gas production [ml $min^{-1}$ $l^{-1}$], hydrogen and carbon dioxide content [%] and redox potential [mV] b) products and residual total sugar [mmol $l^{-1}$].***

The high butyrate and acetate concentrations shown for day 27 in Figure 4-6b were caused by a blockage of nutrient and water supply on day 26, which caused a 12 hour increase in the HRT to 12.5 days without changing the organic loading rate of 16 kg $m^{-3}$ $d^{-1}$. However gas production was unaffected (Figure 4-6a). Once water and nutrient

supply was reinstated and the HRT returned to 15 hours, butyrate and acetate concentrations decreased to 38.9 and 30.5 mmol $l^{-1}$ by day 28. A mass balance for days 27.5 to 31.5 (Table 4-2) shows that 96% of the total TOC in the liquid phase of the reactor were recovered, confirming that all major non-gaseous products are accounted for. The VSS concentration during this period decreased from 3.7 g $l^{-1}$ on day 27 to 1.6 g $l^{-1}$ on day 31.

#### 4.3.3.3 Operation on sugarbeet without sparging

On days 34 to 38 and 41 to 45 the substrate was changed from refined sugar to sugarbeet, with refined sugar fed days 38 to 41. Sugarbeet water extract was fed continuously at approximately 10 g sucrose $l^{-1}$. Additionally, water extracted pulp was injected twice daily during days 34 to 38 and 41 to 43 (section 2.3.2). The residual fibre in the sugarbeet water extract settled in the feed tubing, and the fibre in the pulp repeatedly blocked the overflow pipe. These problems, which could probably be overcome in larger scale operation, together with accidental blocking of water and nutrient supply on day 41 for 12 hours (as on day 26) are most likely the cause of the variability in product concentrations during days 34 to 45 (Figure 4-6b). Ethanol production, which was negligible during operation on refined sugar with and without sparging (concentrations were less than 2.7 mmol $l^{-1}$), increased from day 37 to a maximum concentration of 20.4 mmol $l^{-1}$ on day 43. However, despite the irregular feed rate the hydrogen producing culture did not wash out and > 90% of total sugar was consumed at all times. The average daily gas production rate varied from 1.8 ml $min^{-1}$ $l^{-1}$ to 2.5 ml $min^{-1}$ $l^{-1}$, containing 52 to 57% hydrogen. Daily average yields varied accordingly from 0.6 to 1.2 mol hydrogen per mol hexose converted, with an average of 0.9 ± 0.2 mol hydrogen per mol hexose converted (excluding days 39 to 41, when refined sugar was fed). A mass balance conducted for days 36.0 to 38.5 shows that 98% of filtered TOC was accounted for. Since the VSS fraction here would include sugarbeet pulp as well as microbial biomass, the mass balance of total liquid TOC was not calculated.

### 4.3.4 *Experiment Su10*

#### 4.3.4.1 Operation on refined sugar with sparging

The repeatability of the start-up procedure and operation with nitrogen sparging on refined sugar (Experiment Su9, days 1 to 17) was demonstrated in days 1 to 11 of experiment Su10 (Figure 4-7). To investigate whether hydrogen yields from sugarbeet water extract could be improved through nitrogen sparging, the experiment was sparged throughout, but the substrate was changed to sugarbeet water extract on day 11.

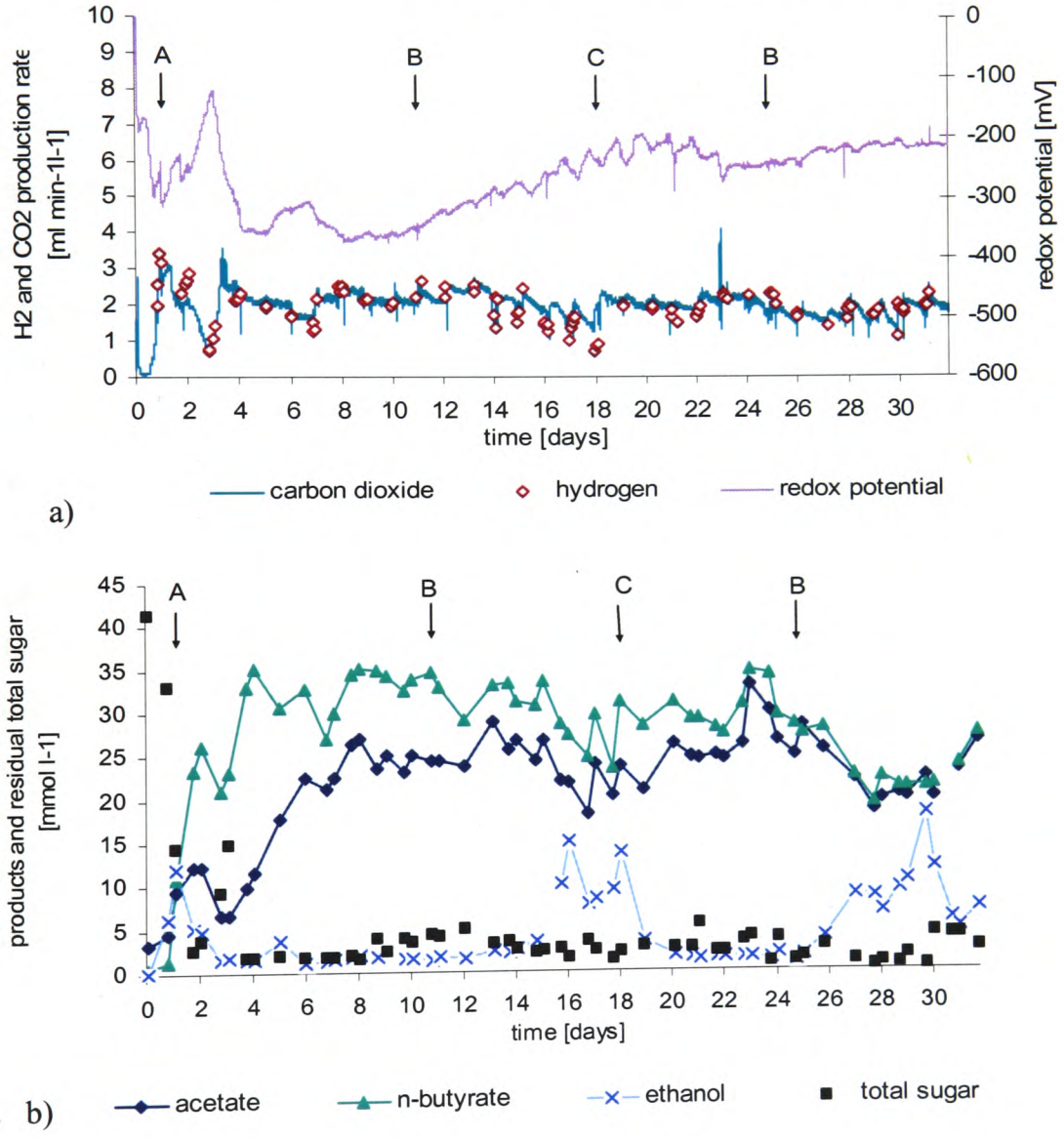



***Figure 4-7. Experiment Su10. Batch start-up and continuous operation on refined sugar (days 1-11 and 18-25) and sugarbeet water extract (days 11-18 and 25-32) without heat-treatment of inoculum and with sparging. Arrow A: continuous operation on refined sugar started. Arrow B: operation on sugarbeet water extract started. Arrow C: operation on refined sugar commenced. a) hydrogen and carbon dioxide production [ml $min^{-1}$ $l^{-1}$] and redox potential [mV] b) products and residual total sugar [mmol $l^{-1}$].***

Observations made were very similar to those in experiment Su9. Hydrogen and carbon dioxide production started as in experiment Su9 after approximately 16 hours (Figure 4-7a). The redox potential decreased to –190 mV within 5 hours and further to –280 mV after 16 hours. This confirms observations from experiment Su9 that use of fresh inoculum and/or nitrogen sparging reduces the long lag time of 60 hours observed in experiment Su8. As in experiment Su9, ethanol and acetate were the main products during batch start-up (day 1), but the butyrate/acetate metabolism became dominant during continuous operation (day 2; Figure 4-7b).

Similar to observations made in experiment Su9, signs of washout appeared on day 3, when the hydrogen production rate and butyrate and acetate concentrations showed a dip, whilst the residual total sugar concentration showed a peak. More clearly than in experiment Su9 the redox potential increased steeply from -300 mV at the beginning of continuous operation on day 1 to –130 mV on day 3 (Figure 4-7a). As in experiment Su9 the culture recovered rapidly, with the redox potential decreasing to –360 mV on day 5. During days 5 to 11 the product concentrations varied little (Figure 4-7b), with the exception of day 7, when the feed rate was accidentally reduced by 30% (causing reduction of the OLR by approximately 30% without significantly affecting the HRT). The average hydrogen production rate was 2.1 ± 0.3 ml $min^{-1}$ $l^{-1}$, giving an average daily yield of 1.7 ± 0.2 mol hydrogen per mol hexose converted. This yield was slightly lower than that of 1.9 ± 0.2 mol per mol hexose converted during days 5 to 17 of experiment Su9 under the same condition (with nitrogen sparging and refined sugar), but still clearly higher than the average daily yield of 1.0 ± 0.1 mol hydrogen per mol hexose converted during days 21 to 32 in experiment 9, when the reactor was not sparged. The average butyrate concentration was 33.9 ± 1.5 mmol $l^{-1}$ (with exception of day 7). Over 90% of substrate was converted and the redox potential remained below –300 mV.

#### 4.3.4.2 Operation on sugarbeet water extract with sparging

On day 11 of experiment Su10 the feed was changed to sugarbeet water extract. Pulp was not added because of the technical problems encountered due to accumulation of fibre in experiment Su9. During days 12 to 18 the hydrogen producing culture appeared to deteriorate gradually. The daily average yield decreased from 2.0 on day 12 to 1.1 mol hydrogen per mol hexose converted on day 18, whilst the redox potential increased

from –350 to –230 mV (Figure 4-7a). Ethanol production started on day 16 (Figure 4-7b), reaching a concentration of 9.5 mmol $l^{-1}$ on day 18. Although the daily average hydrogen yield on day 18 was still 1.1 mol per mol hexose converted, the substrate was changed back to refined sugar in an attempt to halt further deterioration of the culture.

Operation on refined sugar from day 18 to 25 caused an immediate decrease in ethanol concentration to below 2.2 mmol $l^{-1}$ on day 20 and prevented a further decrease in hydrogen production. The average daily hydrogen production rate was 2.0 ± 0.2 ml $min^{-1}$ $l^{-1}$ during days 19 to 25, giving an average yield of 1.6 ± 0.1 mol hydrogen per mol hexose converted, and the redox potential decreased slightly, to –250 mV by day 23. With exception of day 23, when refined sugar inflow was accidentally increased 5 to 10 fold for 30 minutes, average butyrate and acetate concentrations during days 19 to 25 were 29.3 ± 1.2 and 25.0 ± 1.7 mmol $l^{-1}$ respectively. Since hydrogen production appeared to have stabilised by day 25, the substrate was changed back to sugarbeet water extract.

On days 26 and 27, the first 2 days after changing to sugarbeet water extract, the hydrogen producing culture appeared to deteriorate (Figure 4-7b): ethanol production started again, reaching a concentration of 8.8 mmol $l^{-1}$ on day 27, and the daily hydrogen yield decreased to 1.3 mol per mol hexose converted. However, although the ethanol concentration still increased during the following days, reaching a maximum concentration of 18.3 mmol $l^{-1}$ on day 29, the average daily hydrogen production rate increased from 1.7 ml $min^{-1}$ $l^{-1}$ on day 28 to 2.1 ml $min^{-1}$ $l^{-1}$ on day 32, giving an increase in hydrogen yield from 1.5 to 1.9 mol per mol hexose converted with an average of 1.7 ± 0.2 mol per mol hexose converted during days 28 to 32. A mass balance for days 28.0 to 32.0 (Table 4-2) shows that 100.7% of TOC in the liquid phase of the reactor was accounted for.

## 4.4 *Discussion of experiments on sugarbeet and refined sugar*

### *4.4.1 Hydrogen yields*

In batch operation a maximum hydrogen yield of 1.1 mol per mol hexose converted from pulped sugarbeet was achieved in experiment Su5, which is less than the batch yield of 1.4 mol hydrogen per mol hexose converted from starch in experiment St1.

Hydrogen yields obtained during continuous operation are summarised in Table 4-3.

***Table 4-3. Yields [mol hydrogen per mol hexose converted] from refined sugar and sugarbeet water extract (with or without extracted pulp) with and without sparging***

| substrate | sparging | Exp. | days | **Yield** |
|---|---|---|---|---|
| Refined sugar | yes | Su9 | 5 to 17 | **1.9 ± 0.2** |
| Refined sugar | yes | Su10 | 5 to 11 | **1.7 ± 0.2** |
| Sugarbeet water extract | yes | Su10 | 28 to 32 | **1.7 ± 0.2** |
| Refined sugar | no | Su9 | 21 to 32 | **1.0 ± 0.1** |
| Sugarbeet water extract & pulp | no | Su9 | 34 to 38 and 41 to 45 | **0.9 ± 0.2** |

From Table 4-3 can be calculated that through sparging yields from refined sugar and sugarbeet water extract (with or without extracted pulp) were increased by 90%. As similar observations were made on starch (section 3.3.1) and glucose (Mizuno *et al.* 2000a), this effect does not appear to be substrate-specific. From the yield of 1.7 mol hydrogen per mol hexose converted from sugarbeet water extract in experiment Su10 can be calculated that approximately 1900 $m^3$ hydrogen could be produced from sugarbeet grown on one hectare land in the UK. This is based on a sugar yield of 170 g per kg wet beet and a sugarbeet yield of 54 t wet weight per ha (section 1.7.4). According to the Ecology Discovery Foundation New Zealand (2002) 1.8 kg hydrogen provides enough energy for a car to travel 100km, therefore the annual sugarbeet yield from one hectare would provide enough hydrogen fuel for a car to travel 9400 km.

Table 4-4 also shows that yields for operation on sugarbeet water extract (with or without added water-extracted pulp) are comparable to yields obtained from refined sugar. These yields are also within the range of yields from experiments with mixed mesophilic cultures on pure sucrose reported in the literature. Sung *et al.* (2002) for example report yields of 0.8 mol hydrogen per mol hexose from sucrose (17.8 g $l^{-1}$) with heat-treated inoculum in semi-batch mode (pH 5.5, 37°C), Chen and Lin (2003) report a yield of 1.2 mol hydrogen per mol hexose from sucrose (17.8 g $l^{-1}$) at 13.3 h HRT in a

CSTR reactor (pH 6.7, 35°C), and Lin and Jo (2003) report yields of 1.3 mol hydrogen per mol hexose from sucrose (17.8 g $l^{-1}$) at 8 h HRT (pH 6.7, 35°C) in a sequencing batch reactor. Liu and Fang (2002) achieved high yields of 2.2 mol hydrogen per mol hexose from a sucrose rich wastewater (sucrose content of 14.3 g $l^{-1}$), using an acidogenic granular sludge, but there may be difficulties with granular sludge when the substrate contains particulate matter.

### *4.4.2 Start-up*

5 batch experiments and 5 batch start-ups were completed. A summary of batch results on sugarbeet/refined sugar is given in Table 4-4, which shows product concentrations at the end of gas production during batch studies Su1 to Su5 or at the beginning of continuous operation in continuous experiments Su6 to Su10. As for experiments on starch, the beginning of continuous operation was to some extend arbitrary (after start of measurable gas production but limited by working hours), but product concentrations at that point give an indication of the main products during batch start-up.

***Table 4-4. Inoculum pre-treatment, lag phase to gas production and product concentrations [mmol $l^{-1}$] at end of batch studies/start of continuous operation***

| Exp. | Inoculum storage duration [days] | Heat-treatment of inoculum | Lag phase [h] | Butyrate | Acetate | Ethanol |
|---|---|---|---|---|---|---|
| Su1 | 26 | Yes | 19 | 16.1 | 11.1 | 9.9 |
| Su2 | 43 | Yes | 19 | 12.0 | 8.5 | > 25 |
| Su3 | 58 | Yes | 21 | 2.8 | 8.5 | 21.6 |
| Su4 | 65 | Yes | 20 | 9.5 | 10.7 | 20.0 |
| Su5 | 72 | Yes | 9 | 9.6 | 13.4 | 24.9 |
| Su6 | 84 | Yes | 16 | 2.0 | 6.9 | 14.5 |
| Su7 | 100 | Yes | 10 | 0.3 | 5.1 | 15.5 |
| Su8 | 119 | No | 60 | 11.8 | 8.1 | 1.2 |
| Su9 | 21 | No | 16 | 3.2 | 6.6 | 8.9 |
| Su10 | 5 | No | 16 | 10.8 | 9.5 | 11.9 |

Hydrogen production started after a lag phase of 19 to 21 hours (Table 4-4 and section 4.2) in 4 of the 5 batch experiments (Su1 to Su4) with heat treated anaerobic digester sludge and whole sugarbeet pulp. In the batch experiment Su5 and batch start-ups of experiments Su6 and Su7, also with heat treated anaerobic digester sludge and whole sugarbeet pulp, gas production started after a lag phase of just 9 to 16 hours. There is no obvious reason for the shorter lag phase in these experiments, as inoculum for experiments Su1 to Su8 was taken from one sludge stock, which had been collected before experiment Su1. The range of lag phases (9 to 21 hours) observed in experiments

Su1 to Su7 with heat-treated inoculum on sugarbeet extract is very similar to that of 8 to 19 hours observed in experiments St1 to St9 on starch, also with heat treated inoculum. Therefore there is no indication that the lag phase to gas production is substrate specific.

The lag phase to gas production of 16 h in experiments Su9 and Su10 with non heat-treated sludge inoculum stored for <22 days was within the range observed for heat-treated inoculum. This may indicate that heat-treatment does not necessarily shorten the lag phase to hydrogen production when relatively fresh inoculum is used. In experiment Su8, for which the non heat-treated sludge inoculum was stored for 119 days, the lag phase to gas production exceeded with 60 h significantly all others. For experiment St7 in comparison the inoculum was stored even longer, for 147 days, but was heat-treated, and a lag phase of only 17 hours was observed. Heat treatment may therefore shorten the lag phase when inoculum is used which has been stored for longer periods of time. As suggested in section 3.3.2 the reason for this may be that the heat-shock increases the response of germination receptors to the presence of germinants (Johnstone 1994), and possibly also frees germinants by disrupting the sludge matrix. For shorter sludge storage times this mechanism may have less impact, as there may be large numbers of surviving vegetative cells in the non heat treated inoculum, which are able to react to the presence of substrate. Nitrogen sparging may also be an influencing factor on the lag phase. In experiments Su9 and Su10, where the lag phase was significantly shorter (16h) than in experiment Su8 (60h), not only the inoculum was stored for a shorter period, but also sparging commenced as soon as the reactor was filled, whilst experiment Su8 was carried out in self generated gas atmosphere. Similarly, in experiment Su7, for which the inoculum had been stored longest (147days), sparging commenced immediately during start-up. In the experiments on starch with heat-treated inoculum the shortest lag phase of 8 hours was also observed in experiments with nitrogen sparging (experiments St8 and St9). As mentioned before, one expected side effect of nitrogen sparging is a reduction in the dissolved oxygen in the reactor liquid, thus possibly allowing a quicker reduction in the redox potential at start-up (will be discussed in section 4.4.5), which may shorten the lag phase to gas production.

Unlike in experiments with starch (section 3), where butyrate was the main product during all batch start-ups, here butyrate was the main product in only 2 out of 10 batch experiments/start-ups (experiments Su1 and Su8, Table 4-4). In the other 8 experiments ethanol was the main determined product with concentrations of 8.9 to >25 mmol $l^{-1}$ by

the end of gas production in batch experiments or at the beginning of continuous operation. Whilst at the same point in time acetate concentrations in experiments on sugarbeet/refined sugar were with 5.1 to 13.4 mmol $l^{-1}$ similar to those in experiments on starch (3.0 to 15.4 mmol $l^{-1}$) , butyrate concentrations were generally lower on refined sugar/sugarbeet (0.3 to 16.1 mmol $l^{-1}$) than on starch (6.6 to 24.4 mmol $l^{-1}$), and ethanol concentrations were overall higher on sugarbeet/refined sugar (1.2 to >25 mmol $l^{-1}$) than on starch (2.0 to 11.1). Even more so than on starch, ethanol production was therefore identified as a major competing metabolism during batch operation on sugarbeet/refined sugar. Since ethanol was found as a major product in experiments Su4 and Su5, for which the inoculum and the substrate were heat treated, and is also reported a major product in batch experiments with anaerobic sludge inoculum with and without heat treatment on 13 mmol $l^{-1}$ glucose (Oh *et al.* 2004), it can be concluded that ethanol in batch fermentation is produced by spore formers, and that ethanol production is not substrate specific.

As for experiments on starch, no propionate, acetone or butanol production was detected during batch mode in experiments Su1 to Su10. In experiments Su9 and Su10, the only experiments were lactate and formate concentrations were determined, these were also found absent.

Experiments Su9 and Su10 show that stable continuous hydrogen production was established on refined sugar within 5 days when fresh non heat treated sludge was used as inoculum and the culture was initially sparged with nitrogen. This was considerably quicker than start-up procedures for continuous mesophilic hydrogen production from sucrose by a non heat treated mixed culture reported in the literature. Chen and Lin (2001) for example used a start-up procedure which took 56 days, during which the culture was fed semi-continuously with a decreasing HRT from 20 to 2 days, and the produced gas contained just 9% hydrogen. Fang and Liu (2001) increased the sucrose concentration stepwise from 2 g $l^{-1}$ to 12.15 g $l^{-1}$ over 20 days at start-up. Lin and Jo (2003) decreased the retention time in continuous operation from 5 days to 12 hours in 4 steps, each taking 20 days.

### *4.4.3 Butyrate and acetate concentrations during continuous operation*

As reported for experiments on starch, butyrate or acetate were the dominant products during continuous operation. In experiments Su6 and Su7 with heat treated inoculum and start-up on pulped sugarbeet as well as in experiment Su8 with non heat treated inoculum and start-up on refined sugar butyrate concentrations exceeded acetate concentration throughout continuous operation, with a butyrate/acetate ratio of 1.1 to 6.0. However, since all 3 experiments were terminated by washout within 3 to 5 days of continuous operation, general conclusions for continuous operation on refined sugar can not be drawn from this data. If weak homoacetogenesis was present, its effect on hydrogen production would have been insignificant compared to the unknown cause of washout.

Butyrate and acetate concentrations during experiments Su9 and Su10, where stable continuous hydrogen production was achieved, are summarised in Table 4-5 (data from days 16 and 27 of experiment Su9 are excluded as outliers).

***Table 4-5. Butyrate and acetate concentrations during periods in experiments Su9 and Su10***

| Exp. | Days from start-up | Substrate | Hydrogen yield [mol per mol hexose converted] | Acetate [mmol $l^{-1}$] | Butyrate [mmol $l^{-1}$] | Butyrate/ acetate ratio [mol $mol^{-1}$] |
|---|---|---|---|---|---|---|
| Su9 | 5 to 17 | refined sugar | 1.6 to 2.1 | 17.0 to 28.0 | 33.2 to 39.3 | 1.3 to 2.0 |
| | 17 to 34 | refined sugar | 0.7 to 1.2 | 20.4 to 35.2 | 25.0 to 38.9 | 1.0 to 1.4 |
| | 34 to 38 | sugarbeet extract & extracted pulp | 0.8 to 1.2 | 17.6 to 30.9 | 17.0 to 26.5 | 0.7 to 1.2 |
| | 41 to 45 | sugarbeet extract & extracted pulp | 0.6 to 1.2 | 18. to 41.5 | 10.4 to 42.6 | 0.5 to 1.0 |
| Su10 | 5 to 11 | refined sugar | 1.5 to 1.9 | 17.8 to 27.1 | 26.9 to 35.2 | 1.3 to 1.7 |
| | 11 to 18 | sugarbeet extract | 1.1 to 2.0 | 18.1 to 28.9 | 23.5 to 33.6 | 1.2 to 1.4 |
| | 18 to 25 | refined sugar | 1.4 to 1.7 | 20.9 to 33.2 | 27.5 to 34.9 | 1.1 to 1.4 |
| | 25 to 32 | sugarbeet extract | 0.9 to 1.1 | 18.7 to 28.5 | 19.4 to 28.2 | 1.0 to 1.1 |

As the range of butyrate/acetate ratios in Table 4-5 shows, butyrate concentrations exceeded acetate concentrations throughout continuous operation on refined sucrose in

experiment Su9 and throughout experiment Su10. During operation on sugarbeet water extract with addition of extracted pulp in experiment Su9 acetate gradually overtook butyrate concentrations, and the butyrate/acetate ratio reached a minimum of 0.7 mol $mol^{-1}$ on day 31 and 0.5 mol $mol^{-1}$ on day 44 during the two periods.

As observed in experiments on starch, the butyrate/acetate ratio decreased gradually throughout experiments Su9 and Su10, from 2.0 mol $mol^{-1}$ on day 2 to 0.5 mol $mol^{-1}$ on day 44 of experiment Su9 and from 2.1 mol $mol^{-1}$ on day 2 to 1.0 mol $mol^{-1}$ on day 32 of experiment Su10, showing that this occurrence is not substrate specific. A similar gradual decrease of the butyrate/acetate ratio during continuous operation is also reported in the literature from other microbial populations. Sung *et al.* (2002) for example report a decrease from approximately 2 to 0.4 mol $mol^{-1}$ (estimated from data shown in graphs) during hydrogen production from a heat-treated mixed culture on sucrose. Observations by Kataoka *et al.* (1997), who report a decrease from approximately 1.0 to 0.5 mol $mol^{-1}$ during hydrogen production by a pure culture of *Clostridium butyricum*, suggest that the decrease in the butyrate/acetate ratio observed here could be caused by a metabolic shift of the hydrogen producing clostridia.

In experiment Su9 it was also observed that, when sparging was stopped on day 17, the hydrogen production rate decreased from an average of 2.2 ml $min^{-1}$ $l^{-1}$ during days 5 to 17 to 1.2 ml $min^{-1}$ $l^{-1}$ on days 21 to 24. The average butyrate and acetate concentrations during days 21 to 24 however were with 35.6 and 26.3 mmol $l^{-1}$ respectively very similar to those of 35.2 and 23.7 mmol $l^{-1}$ respectively during nitrogen sparging on days 5 to 17. Since over 95% substrate was converted with or without sparging, an increase in the production of hydrogen was expected to be accompanied by increased acetate production at the cost of other products or biomass, but this was not observed. Also, the percentage hydrogen and carbon dioxide determined in the produced gas during days 21 to 24 add up to 100 ± 3%, therefore hydrogen consumption by methanogens can be excluded here. Similarly, Mizuno *et al.* (2000a) reported from a mixed enrichment culture on glucose that hydrogen yields were increased by 68% through sparging, whilst the concentration of butyrate, which was also the main product, was increased by just 10% and that of acetate was not significantly affected.

Observations in experiments on starch suggest that particularly without sparging a significant proportion of the acetate may be produced by homoacetogens and thus be associated with hydrogen consumption rather than hydrogen production. Sparging with nitrogen might therefore have reduced homoacetogenesis and thus increased the proportion of acetate produced in association with hydrogen production. However, whilst presence of homoacetogenesis in experiments on starch was proposed for periods of low hydrogen production, where acetate concentrations were particularly high and clearly exceeding butyrate concentrations, here acid concentrations did not change, and butyrate production exceeded acetate production with or without sparging. It was therefore thought that a significant proportion of hydrogen could have been lost with the effluent.

### *4.4.4 Other fermentation end-products*

The mass balances calculated for periods of experiment Su9 and Su10 with and without sparging on sugarbeet and refined sugar (Table 4-2) showed that 93 to 100% of the liquid end products were accounted for (on carbon basis). Therefore all major end products during these periods were determined. For experiments Su6 to Su8 TOC and VSS were not determined, thus a carbon balance could not be calculated and there is no proof that all major end products have been determined.

As observed for experiments on starch, butanol and acetone production were not detected in any of the experiments on sugarbeet or refined sugar. Lactate was only determined in experiments Su9 and Su10, where it was absent on most days and reached a maximum concentration of only 2 mmol $l^{-1}$.

Apart from butyrate and acetate, ethanol was the only other significant determined liquid end product. As discussed in section 4.4.2 ethanol production was dominant during batch start-up. During continuous operation it generally decreased, but became stronger again (reaching concentrations of 15 to 20 mmol $l^{-1}$) during times of disturbances or irregularities, for example on day 3 of experiment Su7 (Figure 4-3b), when the overflow pipe was blocked, and during periods of feeding with sugarbeet water extract with and without extracted pulp in experiments Su9 (Figure 4-6b) and Su10 (Figure 4-7b), where feed rates were more irregular than on refined sugar. Ethanol as side product of hydrogen production from sucrose has also been reported in the literature. Chen and Lin (2003) reported production of over 15 mmol $l^{-1}$ ethanol from

18.8 g sucrose $l^{-1}$ by a mixed culture in continuous operation at pH 6.7, 35°C and 13.3h HRT, achieving similar yields (max. 1.2 mol hydrogen per mol hexose) to those in experiments Su9 and Su10 during periods without sparging. Interestingly, Ren *et al.* (1995) report stable continuous hydrogen production from molasses by a mixed culture with a dominant acetate/ethanol metabolism at a redox potential of around –250 mV. This metabolism, in which acetate and ethanol are produced in equal amounts, is reported to be more stable than the butyrate/acetate metabolism. However, this has not been reported by other workers. On the contrary, in experiments Su8 to Su10, ethanol production in continuous operation appears to coincide with periods of relatively low butyrate and hydrogen production, for example days 43 and 44 in experiment Su9 (Figure 4-6b) with an average daily yield of 0.6 and 0.7 mol hydrogen per mol hexose converted, and days 16 to 18 in experiment Su10 (Figure 4-7b) with an average daily yield of 1.1 to 1.3 mol hydrogen per mol hexose converted.

### *4.4.5 Redox potential*

The redox potential was only determined in experiments Su8 to Su10.

The redox potential in experiments Su9 and Su10 decreased immediately from the start as in experiments St8 and St9 on starch, which shows that not heat treating the inoculum does not necessarily cause a delay in oxygen consumption. However, the redox potential here decreased only to a minimum of -340 and -310mV respectively by the end of day 1, which is significantly higher than the minimum of -425mV and -450 mV observed in experiments on starch St8 and St9 respectively. As discussed in section 1.6.5, the redox potential of a culture is influenced by a range of parameters. The higher redox potential here could for example be due to the difference in inoculum (here not heat-treated, thus possibly more dominated by non spore-formers). Also, Lin and Jo (2003) report from sequencing batch experiments with waste activated sludge on sucrose (see section 1.6.5) that the redox potential decreased with increasing sucrose loading rates. This agrees with observations made here, that the minimum redox potential in experiments Su9 and Su10 on 10 g $l^{-1}$ sucrose was higher than that in experiments St8 and St9 on 20 g $l^{-1}$ starch.

In experiment Su8 the redox potential did not decrease during the first 20 hours, and only started to drop rapidly after 43 hours. Although delayed by 2 days, the redox

potential then decreased to a minimum of -360 mV at the start of continuous operation, which was similar to the minimum observed for experiments Su9 and Su10. As was discussed for the long lag phase to gas production in section 4.4.2, the delay to decrease of redox potential could be caused by two factors. First of all, the inoculum for experiment Su8 was not heat-treated and had been stored for a comparatively long period (119 days). If predominantly non spore forming facultative anaerobes in the inoculum were responsible for the decrease of the redox potential at the beginning of the experiments on sugarbeet/refined sugar, the longer storage duration of the inoculum used in experiment Su8 might have decreased the survival rate of these non spore formers compared to experiments Su9 and Su10, in which the inoculum was also not heat-treated but stored for only 21 and 5 days respectively. Nitrogen sparging could also have an influence, as the reactor was sparged in experiments St8, St9, Su9 and Su10, but not in experiment Su8. Sparging most likely aids the reduction of the redox potential at the start of the experiment through removal of oxygen from the reactor liquid and headspace.

During continuous hydrogen production in experiments Su9 and Su10 the redox potential ranged from –370 to –150 mV (with the exception of the peak of –130 mV during partial washout on day 3 of experiment Su10; Figure 4-7a). In experiment Su9 no significant difference in the redox potential between sparging and non-sparging conditions could be detected, with an average of -253 ± 37 mV during days 5 to 17 (sparged) and -250 ± 36 mV during days 18 to 33 (non-sparged), which indicates that nitrogen sparging does not determine the redox potential once a continuous hydrogen producing culture is established. Probably due to the disturbances caused by accumulation of fibre, the redox potential varied more strongly during operation on sugarbeet than on sucrose in experiment Su9, but with an average of –255 ± 54 mV was not significantly different to that during operation on refined sugar (-253 ± 37 mV with and –250 ± 36 mV without sparging). On average redox potentials were higher than those of –340 to –320 mV reported by Sung *et al.* (2002, redox reference system not given) for a heat-treated hydrogen producing culture on 17.8 g $l^{-1}$ sucrose and the typical redox potential of around –300 mV reported by Cohen *et al.* (1984, same redox reference system as in this study) for a mixed butyrate/acetate and hydrogen producing culture.

In experiment Su8, as in experiments St9 and St10, onset of washout of the hydrogen producing culture within 5 days of continuous operation was observed. As in experiment St9 the onset of increase in redox potential preceded signs of deterioration of the hydrogen producing culture, as a strong increase in redox potential from -270 to -30 mV on day 6 clearly preceded the decrease of total sugar conversion, suggesting that here the increasing redox potential was possibly the cause for deterioration of the hydrogen producing culture and could have been used as an early indicator. As there were no particular reasons for the washout (no technical problems or changes in the dominant culture metabolism) these observations would support the hypothesis formed in section 3.3.5, that oxygen consuming organisms present in the initial inoculum were out-competed by faster growing clostridia, and the evolving obligate anaerobe culture would then not be able to consume the oxygen added with feed and water during continuous operation, causing an increase in redox potential and sporulation of oxygen sensitive clostridial species. This brings up the question why in experiments Su9 and Su10 the onset of washout and coinciding increase in redox potential on day 3 could be reversed and continuous hydrogen production achieved. It was considered whether there may be a threshold redox value which may initiate sporulation in clostridia. In experiment Su8, where a decrease in substrate conversion to <20% indicated almost complete washout, the redox potential had increased to -30mV by the time substrate conversion started to decrease. During continuous operation in experiments Su9 and Su10 in comparison a maximum redox potential of -130 mV was measured during the partial washout on day 3 of experiment Su10. This may indicate that there exists a threshold redox value between –130 and –30 mV (as measured by the Ag/AgCl electrode as described in section 2.1.3.4) for the given inoculum, substrate and operating conditions. However, more data is required for a clear conclusion.

# 5 Hydrogen production from grass

## *5.1 Grass properties*

The shredded frozen AberDart grass used as substrate for experiments with grass was determined to have a total solids content of 45.8 ± 5.3% (w/w) and an ash content of 3.6 ± 0.3%. This was determined through analysis of 4 replicate samples with the method described in section 2.2.6. For separate samples the α-cellulose was determined as 31.6 and 32.2 % of the total solids (2 samples analysed) and the hemicellulose content as 26.9 and 30.3% of the total solids (2 samples analysed) (Allen *et al.* 1974).

## *5.2 Efficiency of pre-treatments*

Table 5-1 summarises the combinations of grass pre-treatments tested. The chemical pre-treatment was alkaline hydrogen peroxide hydrolysis as described in section 2.3.3.2. The total sugar concentration was estimated with the phenol sulphuric acid assay using sucrose standards as described in section 2.2.9.

***Table 5-1. Grass pre-treatment extraction methods***

| Method No. | Approx. dry matter [1] [g $l^{-1}$] | Physical treatment | Chemical treatment | | Enzymatic treatment | | Total sugar extracted | |
|---|---|---|---|---|---|---|---|---|
| | | | Duration | Temp. | Enzymes added | Duration | [g $l^{-1}$ extract] | [% initial dry matter] |
| 1 | 10 | FWD [2] | - | - | - | - | 1.0 | 9.6 |
| 2 | 30 | FWD | - | - | - | - | 2.5 | 8.1 |
| 3 | 30 | juicer | - | - | - | - | 4.7<br>5.5 | 17.0<br>20.0 |
| 4 | 20 | - | 43 hours | 30°C | - | - | 2.7,<br>3.3 | 13.7<br>16.3 |
| 5 | 20 | - | 45 min | 80°C | - | - | 1.6<br>1.9<br>2.0 | 8.1<br>9.3<br>9.8 |
| 6 | 30 | - | - | - | D740L | 24 hours | 1.7<br>3.3 | 5.4<br>6.8 |
| 7 | 30 | - | - | - | D740L +C013L +D333P | 24 hours | 2.0<br>2.3 | 7.2<br>8.2 |
| 8 | 30 | - | - | - | D740L +C013L +D453P | 24 hours | 2.1<br>4.5 | 7.5<br>16.3 |
| 9 | 30 | FWD | 45 min | 80°C | - | - | 2.4<br>4.5 | 7.8<br>14.5 |
| 10 | 30 | juicer | 45 min | 80°C | - | - | 4.8<br>5.1<br>6.0 | 17.4<br>18.4<br>21.6 |
| 11 | 30 | FWD | 45 min | 80°C | D740L | 24 hours | 7.1<br>7.3 | 22.7<br>23.3 |
| 12 | 30 | FWD | 45 min | 80°C | D740L +C013L +D333P | 24 hours | 8.7<br>10.2 | 28.0<br>32.7 |
| 13 | 30 | FWD | 45 min | 80°C | D740L +C013L +D453P | 24 hours | 9.2<br>10.4 | 29.6<br>33.4 |
| 14 | 30 | juicer | 45 min | 80°C | D740L | 48 hours | 8.9<br>9.0 | 32.4<br>32.5 |
| 15 | 30 | juicer | 45 min | 80°C | D740L +C013L +D453P | 48 hours | 13.3<br>± 1.7 | 48.2<br>± 6.1<br>(4 samples) |

[1] quantity of dry grass suspended in 1 litre water at the start of the extraction method; dry matter estimated from weight of wet grass assumed to have a dry matter content of 45.8%.

[2] FWD = food waste disposer

From Table 5-1 the following general conclusions can be drawn:

- Methods using the domestic all purpose juicer were more efficient than those using the food waste disposer: method 3 was more effective than method 2 (both physical treatment only), and method 10 was more effective than method 9 (combined physical and chemical treatment).
- Physical pre-treatment with the domestic all purpose juicer was the highest yielding single-step treatment: method 3 (juicer only) was more effective than methods 4 to 8 (chemical or enzyme treatment only).
- The combination of physical treatment followed by alkaline peroxide treatment did not clearly yield more total sugar than physical treatment alone: yields from methods 1 to 3 (physical treatment only) are within the range of yields obtained from methods 9 and 10 (physical and chemical treatment)
- The three-step extraction of physical, alkaline peroxide and combined enzyme treatment (method No. 15) clearly achieved the highest yields, giving a yield increase of 140% compared to the most effective single step method (method 3, juicer only) and an increase of 120% compared to the highest yield obtained by combined physical and chemical treatment (method 10).

After the analysis of total sugars as described in section 2.2.9. was completed for experiment Gr1 it was realised that the phenol sulphuric acid assay gave rather different absorbance values for various carbohydrates, as is shown in Figure 5-1.

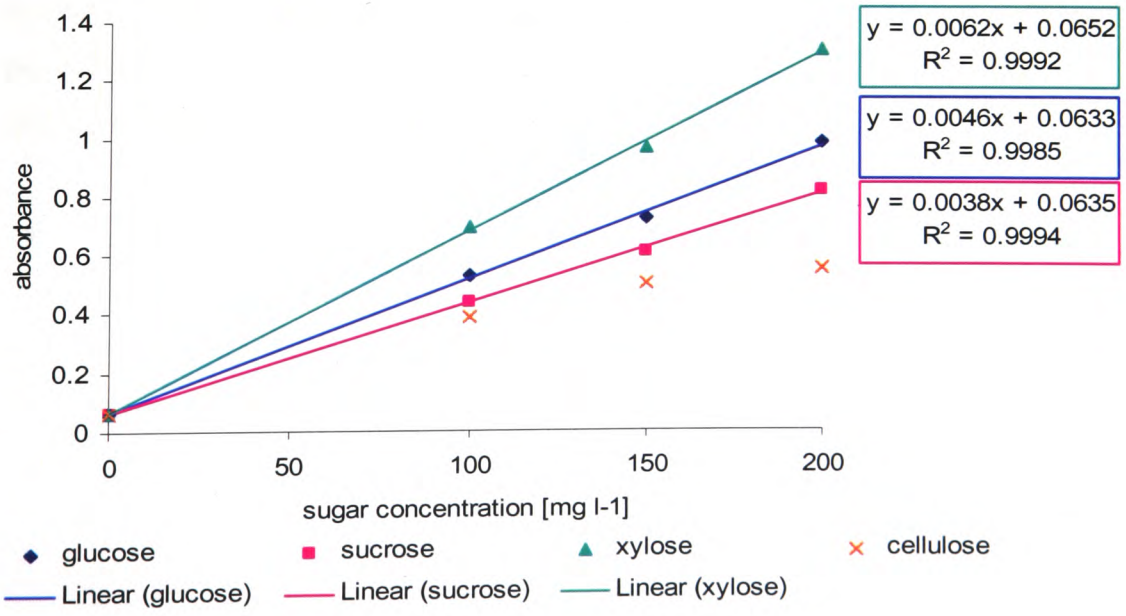



***Figure 5-1. Absorbance of various carbohydrates in phenol sulphuric acid assay.***

Xylose, a pentose, had a stronger absorbance per gram than the hexoses tested. For example, the same standard concentration of 150 mg $l^{-1}$ glucose or sucrose resulted in just 75% and 63% respectively of the absorbance of 150 mg $l^{-1}$ xylose. Cellulose absorbance was weakest and did not increase linearly with increasing concentration. Since it must be assumed that the grass extract contains a combination of poly-, di- and monosaccharides, consisting of both pentoses and hexoses, the proportion of which varies with extraction method, the phenol-sulphuric acid assay is not an accurate method to determine total sugars in the grass extract. Nevertheless, the results should allow an estimate of the relative efficiency of an extraction method.

The method for production of grass extract used as substrate in experiments described in section 2.7.3. was chosen on the basis of the above conclusions from Table 5-1 and for the following reasons:

- Although there was no indication from extraction yields listed in Table 5-1 that alkaline peroxide treatment increased total sugar yields directly, it was thought to remove lignin (Gould 1983) and kill undesired bacteria in the grass, such as lactic acid bacteria, due to the high pH.
- Catalase was added to remove all remaining hydrogen peroxide that might later kill hydrogen producing bacteria in the inoculum.

Due to time restrictions it was investigated if the enzyme treatment of 48 hours in method 15 could be reduced. In a test extraction (using method No. 15) samples for sugar analysis were taken during enzyme treatment after 18.5, 26 and 48 hours. Results of this investigation are shown in Figure 5-2.

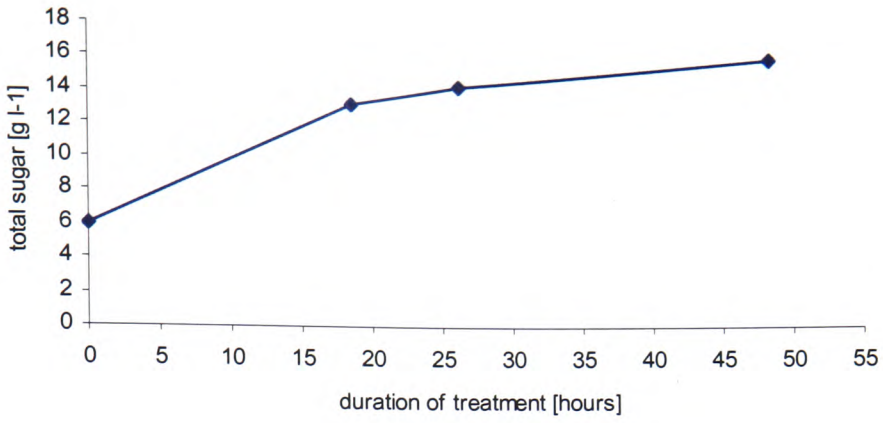



***Figure 5-2. Total sugars extracted during treatment with enzymes D740L, C013L and D453 after physical and chemical pre-treatment ( extraction method No. 15).***

Figure 5-2 shows that of the total sugar extracted by the three step extraction (extraction method No. 15) 38% was extracted by physical and alkaline pre-treatment alone, as the total sugar extracted was 6 g $l^{-1}$ before enzyme treatment, and was increased to 16 g $l^{-1}$ after enzyme treatment. The larger part of the total sugar extracted during enzyme treatment was extracted during the first 18.5 hours, and the amount of sugar extracted during 48 hours was only 10% higher than that extracted during the first 26 hours. From these results it was concluded that enzyme treatment for substrate used in batch experiments could be reduced to 26 hours if time restrictions required it.

The total sugar (estimated with phenol sulphuric acid assay using sucrose standard) in the extracts used as substrate for experiments Gr1 to Gr3 is shown in Table 5-2.

***Table 5-2. Total sugar in grass extract of experiments Gr1 to Gr3***

| Exp. no | Duration of enzyme treatment [h] | Total sugar per litre extract [g $l^{-1}$] | Total sugar extracted per dry grass [% w/w] |
|---|---|---|---|
| Gr1 | 48 | 12.6 | 45.6 |
| | | 12.6 | 45.6 |
| Gr2 | 48 | 9.0 | 32.6 |
| | | 12.0 | 43.5 |
| Gr3 | 26 | 7.5 | 27.1 |
| | | 7.6 | 27.5 |

As can be seen in Table 5-2, the amount of total sugar extracted from the grass substrate of the three experiments was highly variable, and some extract yields are lower than expected from test results of extraction method No. 15 shown in Table 5-1. For experiment Gr3 (26 hour enzyme treatment) only 60% of the total sugar of experiment Gr1 (48 hour enzyme treatment) was extracted, whilst the 2 samples of extract analysed from Gr2 differed widely from each other. It appears that the length of enzyme treatment was more significant than anticipated from the test experiment represented in Figure 5-2. One explanation for this is possibly the difference in sample size. For the test experiment 6g dry grass was extracted in 100 ml water, whilst for the preparation of substrate for experiments Gr1 to Gr3 120g sample was extracted in 2000ml water. It is likely that grass and enzymes were less well mixed in the larger container, reducing enzyme-substrate contact and thus slowing the hydrolysis rate.

Total solids of the grass residue after extraction were determined for experiment Gr1 and found to account for approximately 30% of the initial dry matter. Therefore the estimate for extracted total sugar (Table 5-2) and the grass residue only account for 75% of the initial dry matter. One possible explanation for this is that the phenol sulphuric acid assay strongly underestimated the amount of total sugar extracted. However, since both xylose and glucose have a higher absorbance per mol than sucrose (see Figure 5-1), xylose or glucose concentrations in the sample would have been overestimated rather than underestimated when using sucrose standards. Alternatively, significant amounts of components other than carbohydrates could have been extracted, which were not detected with the phenol-sulphuric acid assay.

A sample of the grass extract used as substrate in experiment Gr3 was analysed for sugars by IGER with HPLC. Results are shown in Table 5-3.

*Table 5-3. Sugars [g $l^{-1}$] in grass extract of experiment Gr3 (analysis by HPLC).*

| Sugars | Per litre extract [g $l^{-1}$] | Per grass dry matter [% w/w] |
|---|---|---|
| fructan | 3.71 | 13.74 |
| sucrose | 1.7 | 6.30 |
| glucose | 7.05 | 26.11 |
| galactose | 2.61 | 9.67 |
| fructose | 2.41 | 8.93 |
| total known sugars | 17.48 | 64.74 |
| unknown sugars (4 peaks) | 4.01 | 14.85 |
| total sugars | 21.49 | 79.59 |

The analysis by HPLC supports the theory that the phenol-sulphuric acid assay underestimates total sugar content of the grass extract, since 17.5 g $l^{-1}$ known soluble sugars were detected in the grass extract (Table 5-3), whilst the phenol sulphuric acid assay gave a total sugar concentration of only 7.5 and 7.6 g $l^{-1}$ (Table 5-2). According to HPLC analysis almost 80% of grass dry matter was extracted. Assuming that the non-structural carbohydrate fraction of the original grass consisted mainly of fructan and sucrose (see section 1.7.5.1), and the fructose in the extract originates from fructan and sucrose in the grass, the total non-structural carbohydrates extracted (=the sum of fructan, sucrose and fructose) account for 29% of the original grass DW, which is feasible for this type of high sugar grass. Assuming that all non-structural carbohydrates were extracted, approximately 71% of the original grass DW are cell wall components, of which approximately 70% were extracted as glucose, galactose and unknown sugars. Considering the grass will also contain some lignin and pectin, this suggests that most of the cell wall carbohydrates in the grass were extracted.

## *5.3 Experiment Gr1 without sparging*

Hydrogen production from grass extract without sparging was investigated in experiment Gr1 ( Figure 5-3). For Figure 2-3a (section 2.1.3.2), showing online versus offline hydrogen data in the range 0-70%, the datapoint at hour 17 in experiment Gr1 (Figure 5-3b) was omitted. The reason for this is that the online sensor was considered to be more affected by a time-delay due to low gas flow rates (<0.1 ml $min^{-1}$) than the off-line measurements. The port for manual gas sampling was directly after the moisture trap, but the gas then passed through the carbon dioxide meter before it entered the hydrogen sensor.

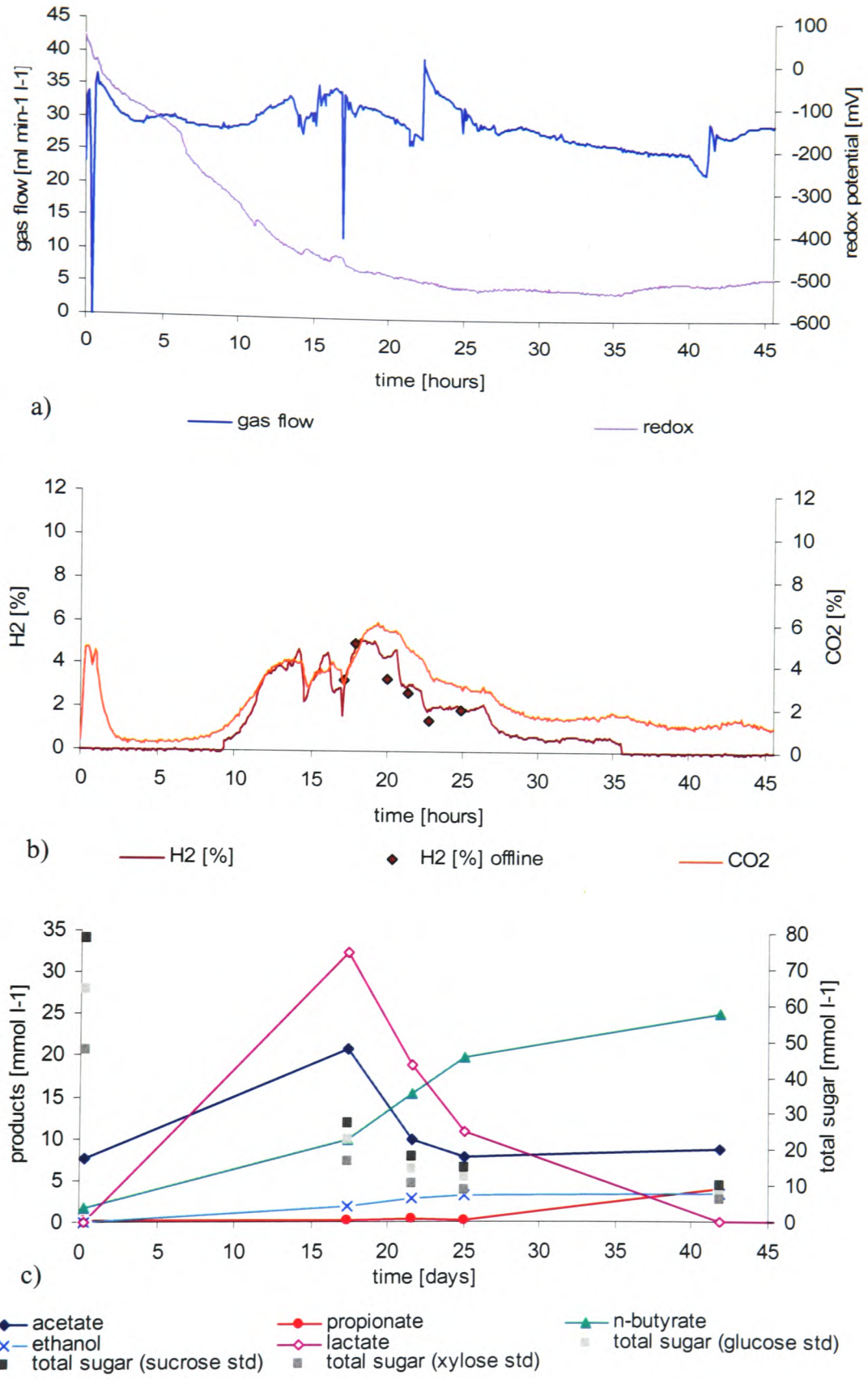



*Figure 5-4. Experiment Gr2. Batch study with sparging. a) total gas flow [ml $min^{-1}l^{-1}$] and redox potential [mV] b) carbon dioxide and hydrogen content [%] c) products and residual total sugar [mmol $l^{-1}$] (glucose, sucrose and xylose standards)*

Total sugar concentrations in mmol $l^{-1}$ in Figures 5-3 and 5-5 were calculated with the assumption that the total sugar consists entirely of hexose units. Figure 5-3a shows that the redox potential decreased from the start and had reached –300 mV by hour 9, when gas production started. Hydrogen and carbon dioxide in the produced gas was first detected after 13 hours (Figure 5-3b), when the gas production reached 0.8 ml $min^{-1}$ $l^{-1}$.

By hour 15, when the redox potential approached its minimum of –500 mV, over 60% of the initial total sugar (determined with the phenol sulphuric acid assay and sucrose standards) was converted and lactate was the main product with a concentration of 27.8 mmol $l^{-1}$ (Figure 5-3c). The experiment shows clearly that lactate is strongly produced at a time when hydrogen production is only just starting. Acetate, butyrate and ethanol concentration were significantly lower with 7.6, 2.4 and 4.4 mmol $l^{-1}$ respectively.

Similar to the intensively monitored batch study with starch (St1; Figure 3-1), the produced gas contained up to 70% hydrogen during the period of increasing gas production, but here hydrogen also still clearly exceeded carbon dioxide content during the peak of gas production hours 19 to 23, whilst in experiment St1 hydrogen and carbon dioxide production became equal during the peak in gas production. Considering the low gas production rate, the time lag between the hydrogen and the carbon dioxide peak here could be due to a lag in the detection system rather than a lag in gas production. However, since the hydrogen peak reached 70% and the carbon dioxide peak 55%, this would give a total of 125% if they had in fact occurred at the same time. Therefore the lag between the hydrogen and carbon dioxide peak is more likely an actual lag in gas production than an effect of delay in the detection.

During hours 15 to 26, when most of the hydrogen was produced, butyrate production increased, reaching a concentration of 15.5 mmol $l^{-1}$ at hour 26. Lactate levels continued to rise at first, reaching a concentration of 35.1 mmol $l^{-1}$ at hour 21, but lactate was consumed thereafter. Changes in acetate, ethanol and propionate concentrations were negligible.

From hour 21 hydrogen production decreased, reaching negligible levels by hour 36, whilst the redox potential increased to around –330mV. Butyrate production continued, reaching a concentration of 25.1 mmol $l^{-1}$ at hour 40.

A total of 460 ml hydrogen per litre reactor volume (2.3 l reactor volume) was produced during this experiment, giving a yield of 0.019 $m^3$ $H_2$ per kg dry grass. Over 90% of the hydrogen was produced after hour 15, at which point over 60% of the total sugars was already consumed.

## *5.4 Experiments Gr2 and Gr3 with sparging*

Hydrogen production from grass extract with nitrogen sparging was investigated in 2 repeat experiments.

### *5.4.1 Experiment Gr2*

Data for experiment Gr2 are shown in Figure 5-4. As in experiment Gr1 hydrogen production started after a lag phase of 9 hours, by which point the redox potential had decreased to –320 mV. During hours 12 to 17 strong foaming occurred, which caused loss of reactor liquid, approximately 6 to 7% of reactor volume, through the stirrer shaft and gas tubing to the Drechsel bottle, probably leading to an underestimate of the total hydrogen production. The redox potential decreased to –480 mV during that time.

The total sugar in this experiment was analysed with 3 different standards: glucose, sucrose and xylose (data for these standards were shown in Figure 5-1). As a consequence of the range of absorbance of the standards shown in Figure 5-1, Figure 8-4c shows that the total sugar values depended on the reference carbohydrate. For example, the estimate of the initial total sugar concentration ranges from 47 mmol $l^{-1}$ assuming 100% xylose to 64 mmol $l^{-1}$ assuming 100% sucrose. Table 5-3 shows that the substrate for experiment Gr3 contained 6.3% sucrose, 26% glucose, a maximum of 15% pentose (in form of unknown sugars) plus 32% other hexoses (fructan, galactose and fructose), for which absorbance in the phenol sulphuric acid assay was not determined. Considering this mix of carbohydrates and the fact that these might be converted at different rates, thus the substrate composition change throughout the batch experiment, total sugar estimates of the grass extract can only be taken as indicators of substrate conversion.

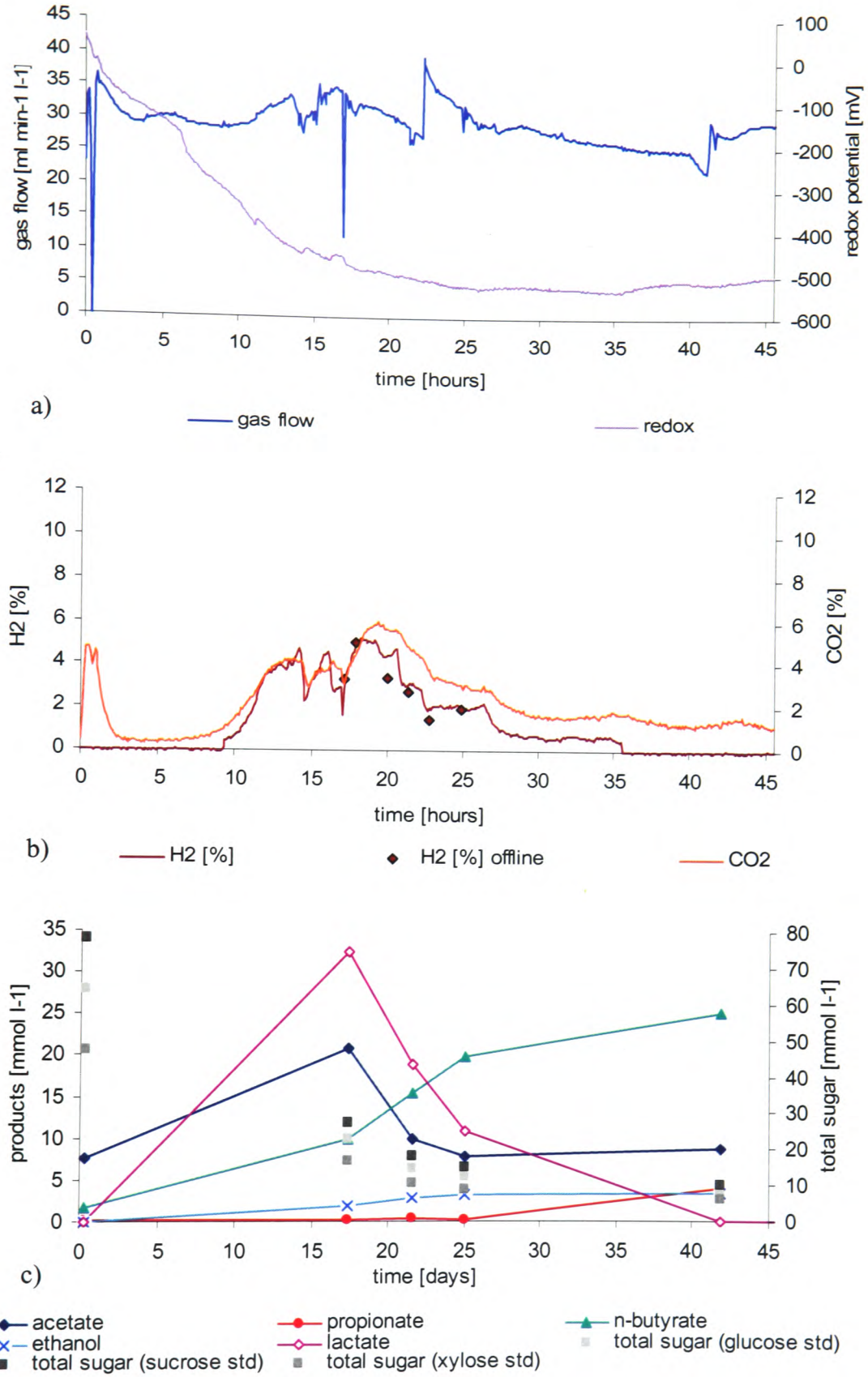



*Figure 5-4. Experiment Gr2. Batch study with sparging. a) total gas flow [ml $min^{-1}l^{-1}$] and redox potential [mV] b) carbon dioxide and hydrogen content [%] c) products and residual total sugar [mmol $l^{-1}$] (glucose, sucrose and xylose standards)*

As observed in experiment Gr1 without sparging, lactate was the main product with a concentration of 32.4 mmol $l^{-1}$ (Figure 5-4c) by hour 17. 13.2 mmol $l^{-1}$ acetate and 8.1 mmol $l^{-1}$ butyrate were also produced, whilst no ethanol or propionate was detected.

The peak in hydrogen production occurred most likely during the period of foaming. Once antifoam was injected and the gas tubing cleared at hour 17, the hydrogen content of the exiting gas was 5% and decreased gradually to 0 by hour 36. Throughout this period the carbon dioxide exceeded the hydrogen content of the produced gas. From hour 17 butyrate was the main product, increasing to a concentration of 25.3 mmol $l^{-1}$ by hour 42. This was accompanied by production of 4 mmol $l^{-1}$ propionate, whilst all lactate and 12 mmol $l^{-1}$ acetate were converted.

1130 ml hydrogen per litre reactor volume was produced, giving a yield of 0.047 $m^3$ $H_2$ per kg dry grass and a yield increase from non-sparging of 147%, even with possible hydrogen loss due to foaming.

### *5.4.2 Experiment Gr3*

Data from experiment Gr3 are shown in Figure 5-5. Reactor samples taken at hour 0 and 42 of experiment Gr3 were sent off for analysis of carbohydrate content by HPLC as shown in Table 5-4. Data for the grass extract used as substrate for this experiment is included in Table 5-4 for comparison, but has already been shown in Table 5-3.

***Table 5-4. Sugars [g $l^{-1}$] in grass extract and samples of Experiment Gr3***

| | Grass extract | reactor sample hour 0 | reactor sample hour 42 |
|---|---|---|---|
| fructan | 3.71 | 1.97 | 0 |
| sucrose | 1.7 | 1.89 | 0 |
| glucose | 7.05 | 5.02 | 0 |
| galactose | 2.61 | 2.07 | 0 |
| fructose | 2.41 | 1.51 | 0 |
| total known sugars | 17.48 | 12.46 | 0 |
| unknown sugars (4 peaks) | 4.01 | 0.79 | 2.28 |
| neutral polymers | 0 | 2.88 | 2.17 |
| Sum of determined products | 21.49 | 16.13 | 4.45 |

Table 5-4 shows that a minimum of 12.46 g $l^{-1}$ water soluble carbohydrates (total known sugars) were available at the beginning of the experiment. The dilution from 17.48 g $l^{-1}$

total known sugars in the grass extract to 12.48 g $l^{-1}$ total known sugars in the reactor at the start of the experiment agrees with dilution of 1800 ml grass extract to a total working reactor volume of 2300 ml (by addition of sludge and nutrients). However, it can not be explained why the fructan and fructose concentrations decreased by 47% and 37% respectively in the dilution, whilst the sucrose concentration increased. Most unexpectedly, the determined unknown sugar concentration decreased by 80%, which may need to be considered an instrument error, particularly as the unknown sugar concentration then increases again between hours 0 and 42. The addition of neutral polymers originated most likely from the sludge inoculum and are not necessarily carbohydrates (Morris 2004).

Hydrogen production started after a lag phase of 10 hours (Figure 5-5b) The redox potential at this point had decreased to –200 mV (Figure 5-5a), which was higher than that of -480 mV and -300 mV at the start of hydrogen production in experiments Gr1 and Gr2 respectively. As in experiment Gr1 the exiting gas contained more hydrogen than carbon dioxide up to the peak in gas production at hour 19, at which point the redox potential had decreased to –470 mV.

As in both previous experiments, lactate was the main product at first, reaching a concentration of 25.1 mmol $l^{-1}$ at hour 19. Acetate and butyrate were produced in lower concentrations of 5.0 and 8.2 mmol $l^{-1}$ respectively at hour 19.

After the gas peak at hour 19 the hydrogen and carbon dioxide content of the exiting gas decreased gradually and, as observed in experiments Gr1 and Gr2, carbon dioxide now exceeded hydrogen production, which ceased at hour 38. Butyrate was the main product from hour 19, its concentration increasing to 28.4 mmol $l^{-1}$ at hour 42, whilst all lactate and 2 mmol $l^{-1}$ acetate was converted. Small amounts of ethanol (2 mmol $l^{-1}$ at hour 42) and no proprionate was produced. The redox potential remained steady at –480 to –520 mV. Table 5-4 shows that all known carbohydrate was converted by hour 42. There is no indication in the literature to date whether fructan can be converted to hydrogen (section 1.7.5.1). Whilst experiment Gr3 does not prove that fructan can be converted to hydrogen, it shows that the mixed microflora was able to hydrolyse all fructan in the substrate.

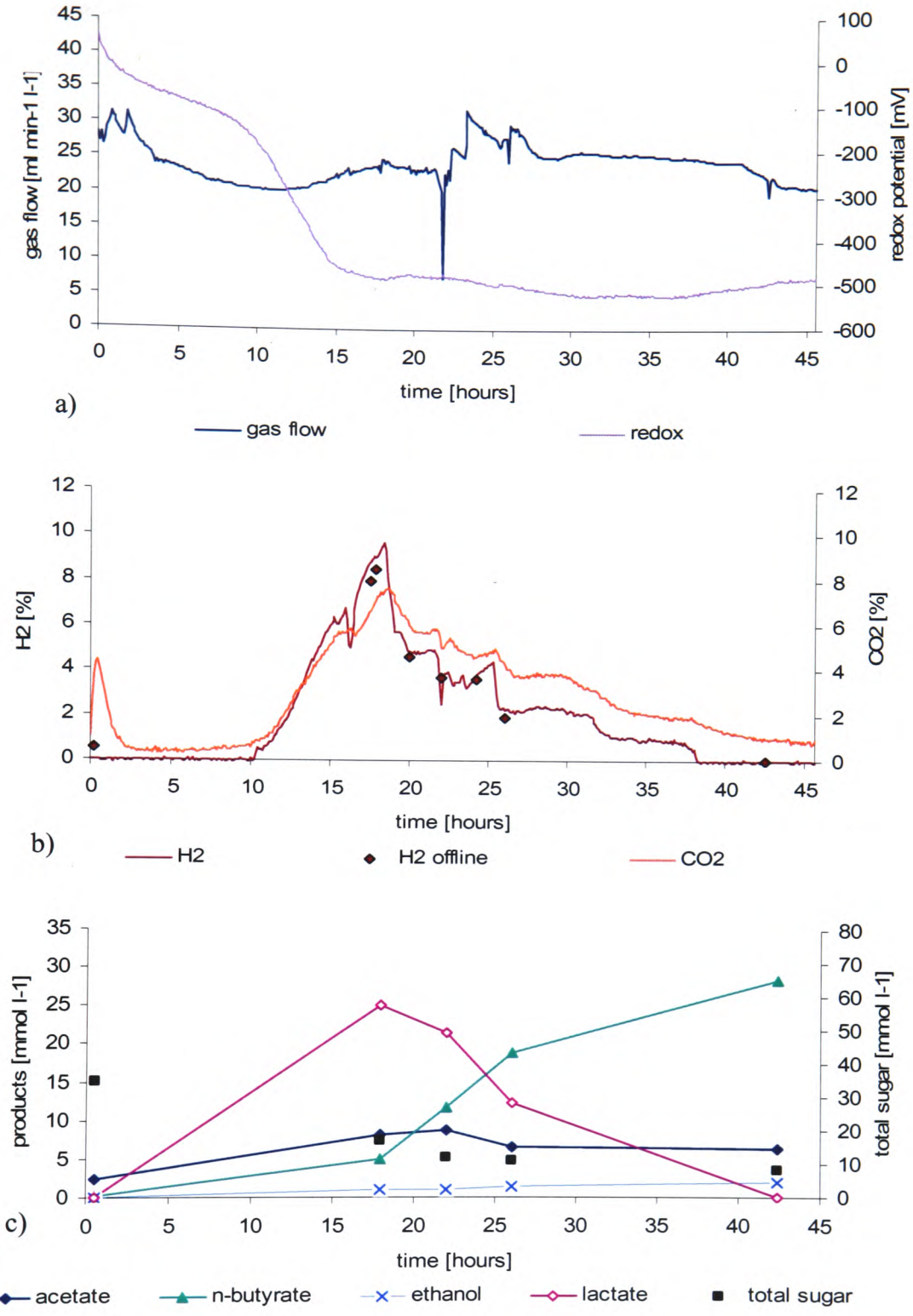



***Figure 5-5. Experiment Gr3. Batch study with $N_2$ sparging a) total gas flow [ml $min^{-1}l^{-1}$] and redox potential [mV] b) carbon dioxide and hydrogen content [%] c) products and residual total sugar [mmol $l^{-1}$] (sucrose standard).***

1300 ml hydrogen per litre reactor volume was produced in experiment Gr3, giving a yield of 0.054 $m^3$ $H_2$ per kg dry grass. Assuming that the 12.5 g $l^{-1}$ known sugars

converted during hours 0 to 42 (Table 5-4) are a reasonable estimate for the amount of total carbohydrate converted (i.e. only known sugars were converted to hydrogen), and assuming that all carbohydrate is hexose, this would give a hydrogen yield of 0.8 mol per mol hexose converted.

## *5.5 Discussion of experiments with grass*

### *5.5.1 Hydrogen yields*

The experiments show that fermentative hydrogen production from grass extract is possible. A yield of 0.019 $m^3$ $H_2$ per kg dry grass was achieved without sparging, and this was improved by a factor of 2.8 to 0.054 $m^3$ $H_2$ per kg dry grass with nitrogen sparging at a rate of 20 to 30 ml $min^{-1}$ $l^{-1}$. Assuming a rye grass yield of 12 t $ha^{-1}$ in the UK (section 1.7.5), this would give a hydrogen yield of 648 $m^3$ per ha.

Comparison of total sugar values obtained with the phenol sulphuric acid assay using a range of carbohydrate standards showed that this method is not easily interpreted for total sugar analysis in substrates with unknown carbohydrate composition such as the grass extract. This was also confirmed by analysis of sugars with HPLC for grass extract in experiment Gr3, which gave a significantly higher sugar concentration. Therefore yield calculations in terms of mol hydrogen per mol hexose converted were not possible for experiments Gr1 and Gr2. For Gr3 a yields of 0.8 mol hydrogen per mol hexose converted was estimated from HPLC data. This hydrogen yield is low compared to that of 1.4 mol per mol hexose converted in the batch experiment St1 on starch without sparging, but is similar to the estimated yields of < 1 mol per mol hexose converted in experiments Su1 to Su4 on sugarbeet.

### *5.5.2 Other fermentation end products*

Maximum product concentrations observed in the 3 experiments on grass are summarised in Table 5-5.

***Table 5-5. Maximum product concentrations assayed [mmol $l^{-1}$] in experiments Gr1, Gr2 and Gr3***

| Exp. | butyrate | acetate | lactate | ethanol | propionate |
|---|---|---|---|---|---|
| Gr1 | 25.1 | 9.8 | 35.1 | 5.5 | 6.7 |
| Gr2 | 25.3 | 20.8 | 32.4 | 1.9 | 0.2 |
| Gr3 | 28.4 | 8.8 | 25.1 | 1.9 | 0.9 |

Table 5-5 shows that butyrate and lactate were the main products, with lactate dominant in experiments Gr1 and Gr2, and butyrate dominant in experiment Gr3. Whilst acetate concentrations were similar to those observed in batch mode of experiments on starch and sugarbeet/refined sugar, butyrate concentrations were close to the maximum butyrate concentrations observed in experiments on starch (ranging from 6.6 to 26.6 mmol $l^{-1}$; Table 3-2) and exceeded those of 0.3 to 16.1 mmol $l^{-1}$ observed in experiments on sugarbeet/refined sugar. Ethanol concentrations were low compared to experiments on starch and sugarbeet/refined sugar (ethanol concentrations ranging from 1.2 to >25 mmol $l^{-1}$). No acetone or butanol was produced.

Table 5-5 shows that sparging with nitrogen, which significantly increased the hydrogen yield, did not clearly affect the maximum concentration of butyrate, acetate and lactate. Lactate was the dominant product during gas production with or without sparging, and butyrate exceeded acetate production at the end of gas production in all three experiments (Figures 5-3c, 5-4c and 5-5c). However, the side products ethanol and propionate were both clearly lower in experiments Gr2 and Gr3 with nitrogen sparging.

Compared to the batch study St1 on starch, where the determined products were produced simultaneously and steadily during the period of gas production (Figure 3-1), in experiments on grass a clear sequence of product formation can be detected. Lactate production was dominant up to the peak in hydrogen production (Figures 5-3c, 5-4c and 5-5c), coinciding with the period of the highest carbohydrate conversion rate. Since grass contains naturally high numbers of lactic acid bacteria, which are chiefly responsible for acidification of the grass during silage making, it was thought that the lactic acid bacteria present in the reactor could have been supplied with the grass extract. However, there was no lactate present at the beginning of any of the experiments, and vegetative cells of the lactic acid bacteria could have been killed by the alkali pre-treatment. Considering that dominant lactate production was also observed during batch start-up in experiments St8 and St9 on starch, it is likely that the lactic acid producing bacteria originated from the anaerobic digester sludge. As the sludge had been heat-treated to 110°C, they are most likely spore-formers. It is possible that lactate production, which is not associated with hydrogen production (equation 6), but competes with hydrogen producers for substrate, may also have been present during the

lag phase of batch experiments on sucrose, possibly explaining the low hydrogen yields observed in all 5 batch experiments with sugarbeet.

Acetate was present in comparatively high concentrations of 2.3 to 7.6 mmol $l^{-1}$ at the very beginning (time 0) of all three experiments, possibly contained in the grass extract. Its production was simultaneous with but weaker than lactate production, and concentrations reached a maximum at the peak in gas production. Butyrate production lagged behind lactate and acetate production, and butyrate concentrations increased most strongly during the period from the peak to end of hydrogen production. Curiously, lactate and acetate concentrations decreased after the peak in gas production, which indicates that they were partly converted during this time.

The question arises, what lactate and acetate could have been converted to. Little information is available from the literature about fermentation of lactate in a mixed hydrogen producing culture. One known metabolism is the conversion of lactate to propionate and acetate by some clostridial species (section 1.3.4), but in experiments Gr2 and Gr3 propionate was not produced in significant quantities. A not well understood metabolism, converting lactate and acetate to butyrate was recently proposed by Duncan *et al.* (2004) for isolates from human faeces. 16S rRNA sequencing showed that these isolated belonged to clostridial cluster XIVa, a highly abundant bacterial group in human faeces. Conversion of lactate to butyrate in batch experiments was only observed once glucose in the medium was depleted (Duncan *et al.* 2004), which agrees with observations in experiments Gr1 to Gr3, where the larger part of the substrate was converted to lactate before lactate conversion started. Duncan *et al.* (2004) propose that in this metabolism 4 mol lactate and 2 mol acetate are converted to 3 mol butyrate, 2 mol hydrogen and 4 mol carbon dioxide. Assuming 2 mol lactate was initially produced per mol hexose (equation 6), this would give an overall hydrogen yield of 1 mol hydrogen per mol hexose converted. Therefore direct conversion of hexose to butyrate as in equation 2 would overall be preferable for hydrogen production.

### 5.5.3 *Redox potential*

As observed in batch operation on starch and sugarbeet/refined sugar, the redox potential decreased immediately from the start in all three experiments. In experiment Gr1 measurable hydrogen production started after 13 hours, at which point the redox potential had decreased to -480 mV. In experiments Gr2 and Gr3 with nitrogen sparging measurable hydrogen production started earlier, after 9 and 10 hours respectively, whilst the redox potential had only decreased to -300 and -200 mV respectively. By hour 13 the redox potential in experiments Gr2 and Gr3 with sparging had still not decreased to the level observed in experiment Gr1 with -440 and -400 mV respectively. This suggests that whilst sparging in experiment Gr2 and Gr3 shortened the lag phase to detection of hydrogen production (due to higher gas flow rates), sparging did not accelerate the decrease in redox potential.

The redox potential reached a minimum of –500 to –530 mV in all 3 experiments, which is lower than in experiments with starch or sucrose. This suggests that the minimum redox potential during batch mode may be substrate specific. As discussed in section 1.4.5. hydrogen production is more commonly associated with redox potentials of –250 to –300 mV. However, similarly low redox potentials of –500 to –550 mV have been reported during continuous hydrogen production from *Clostridium butyricum* by Kataoka *et al.* (1997, method not given). Lin and Jo (2003, method not given) also observed redox potentials as low as –590 mV during fed-batch hydrogen production from a mixed culture at high organic loading rates (40 g COD $l^{-1}$ $d^{-1}$).

# 6 Conclusions

- It was shown for the first time that hydrogen can be produced continuously from a particulate starch industry co-product and sugarbeet and in batch from grass extract. On starch, hydrogen was produced continuously for 18 days with an average yield of 1.9 mol hydrogen per mol hexose converted. On refined sugar and sugarbeet water extract hydrogen was produced continuously for 40 days with average daily yields of 0.9 to 1.9 mol hydrogen per mol hexose converted. On both substrates there was no indication of a decline in hydrogen production when the experiments were terminated and it can be assumed that hydrogen production could have continued further.

- Reduction of the hydrogen partial pressure through nitrogen sparging was demonstrated to be the most influential of the operational parameters tested on the starch substrate. Sparging improved hydrogen yields on all three substrates and increased the stability of hydrogen production in continuous operation on refined sugar/sugarbeet extract and starch. On starch, highest yields achieved in self generated gas atmosphere and continuous operation were equal at pH 4.5 and 5.2, with an average 1.3 mol hydrogen per mol hexose converted for a minimum of 3 to 5 days. This was increased to an average yield of 1.9 mol hydrogen per mol hexose converted for 18 days through sparging with nitrogen at 10-20 times the hydrogen production rate. Similarly, in continuous operation in self generated gas atmosphere on sugarbeet water extract and water extracted pulp average daily hydrogen yields of $0.9 \pm 0.2$ mol per mol hexose converted were achieved, and $1.0 \pm 0.1$ mol per mol hexose converted on refined sugar. Through sparging at a rate of 10-20 times the hydrogen production rate these yields were improved to 1.7 mol per mol hexose converted on sugarbeet water extract and 1.7 to 1.9 mol per mol hexose converted on refined sugar. On grass, yields could only be estimated assuming all carbohydrate was hexose. However, it was shown that a batch yield of 0.019 $m^3$ $H_2$ per kg dry grass was achieved without sparging, and that this could be improved to 0.054 $m^3$ $H_2$ per kg dry grass with nitrogen sparging at a rate of 10 times the maximum hydrogen production rate.

- On all three substrates hydrogen production was more closely related to butyrate than acetate levels, which partly explains why the hydrogen yields obtained are far off the theoretical maximum of 4 mol per mol hexose converted in association with acetate production. Continuous hydrogen production on starch and sugarbeet/refined sugar was generally associated with butyrate/acetate ratios of between 1 and 2 mol $mol^{-1}$. In continuous operation on starch, strong dominant acetate production often occurred during times of relatively low hydrogen yields. Homoacetogenesis, as described by equation 10, was suggested to be the most likely explanation. On starch, signs of homoacetogenesis were present at pH 4.5 and 5.2, and 30, 32 and 35°C, at 18, 15 and 12 h HRT, on 10 g $l^{-1}$ substrate without sparging and on 20 g $l^{-1}$ substrate with sparging. On sugarbeet/refined sugar signs of homoacetogenesis were not observed.

- Methane production, a possible constraint to net hydrogen production, was not observed at pH < 6.0. Acetone/butanol production, which was expected to be a significant problem (since it is thought that clostridia, which have been commercially exploited for solvent production, are the dominant hydrogen producers in anaerobic digester sludge), was not observed. Lactate, ethanol and propionate production were found to be the main non hydrogen producing metabolisms in addition to homoacetogenesis. Since they are competing with the hydrogen producing metabolism for the carbohydrate substrate, their presence partly explains low hydrogen yields. These products are all reduced end-products, which were expected to increase with increasing hydrogen levels (Harper and Pohland 1986; Boone and Mah 1987; section 1.6.1). The fact that their production occurred in experiments with and without nitrogen sparging suggests that hydrogen stripping can still be improved to give molecular hydrogen production an advantage over production of reduced end products.

- Lactate was assayed for in 3 batch (all on grass) and 4 continuous (2 on starch, 2 on sugarbeet) experiments. Lactate production was observed particularly during batch operation, as it was the dominant determined product during gas production in all 3 batch experiments on grass extract and during batch start-up in experiments St8 and St9 on starch. The inoculum in these experiments was heat treated, therefore the lactate producers could be supplied with the substrate (starch and grass), or spore

formers present in the inoculum. In experiments St8 and St9 lactate production continued during continuous operation, but ceased to be dominant, and lactate was not found present in experiments Su9 and Su10 on sugarbeet, which suggests that lactate production could be overcome through continuous operation.

- Ethanol production was also generally stronger during batch than continuous operation, and was observed with all three substrates but was weakest on grass. In batch experiments on sugarbeet, where lactate was not assayed, ethanol was found the dominant determined product, exceeding acetate and butyrate production on a molar basis. Ethanol production also increased during continuous operation on sugarbeet at times of variable feed rate (Su10) and appears to be disadvantaged by low feed concentrations. Ethanol production was observed in experiment Su5, for which the inoculum was heated to >90°C and the sugarbeet pulp was autoclaved. Ethanol production was therefore not inhibited by heat treatment of the inoculum and most likely caused by spore-formers.

- Significant propionate production was observed in experiments on starch and experiment Gr 1 on grass. Propionate production during continuous operation on starch appeared to develop gradually. It was also observed that shortening of the HRT from 18 to 12 hours reduced propionate production slightly, which may indicate that the propionate producers have a slower growth rate than clostridia.

- Continuous hydrogen production was achieved from anaerobic digester sludge inoculum with heat treatment on starch and without heat treatment on sugarbeet/refined sugar. Clear conclusions could not be drawn on the effect of heat pre-treatment on hydrogen yields and bacterial metabolisms. With and without heat treatment, a rapid decrease in redox potential preceded the onset of measurable gas production in start-ups. It was proposed that this strong decrease in redox potential indicated the presence of oxygen consuming and therefore facultative anaerobic microorganisms. Since this was also observed in experiments where the inoculum was heat-treated, these organisms were proposed to be spore formers, possibly bacilli. Experiments on sugarbeet/refined sucrose indicate that without heat treatment of the inoculum the lag phase to the decrease in redox potential and to onset of measurable gas production was shorter for shorter inoculum storage

duration and/or nitrogen sparging. A 43 hour lag phase to decrease in redox potential and a 60 hour lag phase to gas production was observed when the inoculum had been stored for 119 days and the reactor was not sparged, compared to instant decrease in redox potential (at time 0) and 16 hour lag phase to gas production when sludge was stored for 5 or 21 days and the reactor was sparged. When the inoculum was heat treated, the decrease in redox potential started instantly (as soon as the reactor was sealed) and a lag phase to gas production of 8 to 21 hours was observed independent of inoculum storage duration or substrate type. However, as for non heat-treated inoculum, the lag phase to gas production was possibly shortened through nitrogen sparging (sparging experiments had lag phases at the lower end of the range).

- The redox potential decreased rapidly during batch start-up. The lowest redox potential observed throughout experiments appeared to be substrate specific, reaching -425 and -450 mV on starch, -360 to -310 mV on refined sugar and -530 to -500 mV on grass. In continuous operation the lowest redox potential was observed during batch start-up and beginning of continuous operation, and a decrease in hydrogen production was generally associated with an increase in redox potential. In different experiments change in redox potential was observed to precede, coincide or follow a change in hydrogen production. From the data obtained it is therefore not possible to conclude if change in redox potential is cause or effect of a change in hydrogen production. No general redox value above which hydrogen production irreversibly ceases could be established. For experiments on sugarbeet/refined sugar a threshold redox value between –130 and –30 mV (relative to the Ag/AgCl reference system) was proposed.

- Of the tested grass extraction methods a three step extraction entailing physical pre-treatment with a multipurpose juice extractor followed by alkaline peroxide treatment followed by enzyme treatment with ferulic acid esterase, cellulase and xylanase was found to give highest total sugar yields. Analysis with HPLC of one extract obtained by this method showed that almost 80% of grass dry matter was extracted.

# 7 Further work

- Optimisation of the nutrient solution should be investigated. The aim in the experiments here was to operate in carbon limitation to achieve maximum carbohydrate conversion rates, therefore other nutrients were possibly supplied far in excess. Studies by Heyndrickx *et al.* (1990) for example, which showed high hydrogen yields in phosphate limitation (see section 1.7.7), indicate that certain nutrient limitations would aid hydrogen production. Also, in the industrial solvent production process, maize meal and potato mash could be fermented without addition of nutrients. Some nutrients, particularly the trace elements added in the mineral solution, may not be required at all when complex substrates such as pulped sugarbeet are fed. Increase in substrate concentration should also be further investigated, since it would also operating costs.

- Analysis of the microbial community, for example by 16S rDNA, at times of strong hydrogen production, but also at times of strong acetate (with low hydrogen), ethanol, lactate or propionate production, should provide valuable information about the cause for different dominant end products. If different metabolic end products are a consequence of different microbial populations, environmental conditions or inoculum pre-treatment could be changed to disadvantage undesired microorganisms, particularly if these organisms were identified and their growth characteristics known.

- To demonstrate homoacetogenesis, tracer studies with isotopically labelled carbon dioxide could show if and what percentage of acetate is produced from hydrogen and carbon dioxide. Since in homoacetogenesis carbon dioxide would not only be reduced to acetate, but also incorporated into the biomass, homoacetogens could also be identified through stable isotope probing, bearing in mind that some methanogens might also incorporate the carbon from the isotopically labelled carbon dioxide or formed acetate into their biomass.

- Thermophilic operation could be investigated. A thermophilic mixed culture would consist of a different microbial community to a mesophilic culture, therefore some of the problems, such as homoacetogenesis or lactate production, may not be encountered. However, from energetic aspects, thermophilic operation is most

appropriate for fermenting already heated waste products or products from processes with waste heat. For hydrogen production from biomass crops it would only be suitable if the gain through increase in hydrogen yield compared to mesophilic operation would exceed the additional heating costs including capital costs of heat exchangers.

- For scale-up, calculations would be necessary to investigate the energy balance and life cycle analysis of the whole process, since fermentative hydrogen production is only feasible if the energy and monetary value of the produced hydrogen exceeds the energy requirements and costs of the whole production process including capital items. Therefore crop production costs (planting, fertiliser, pesticide, harvesting, transport and storage costs), substrate pre-treatment costs (physical, chemical, biological pre-treatments) and European agricultural policies would need to be considered for the substrate selection. For a farm-scale plant in any location in the UK it would also be important that substrate would be available all year round. Therefore hydrogen production from a range of other fermentable biomass crops, such as miscanthus or sweet sorghum, should be investigated.

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# 9 Appendix

*Figure 9-1. Photo of reactor A before start-up*

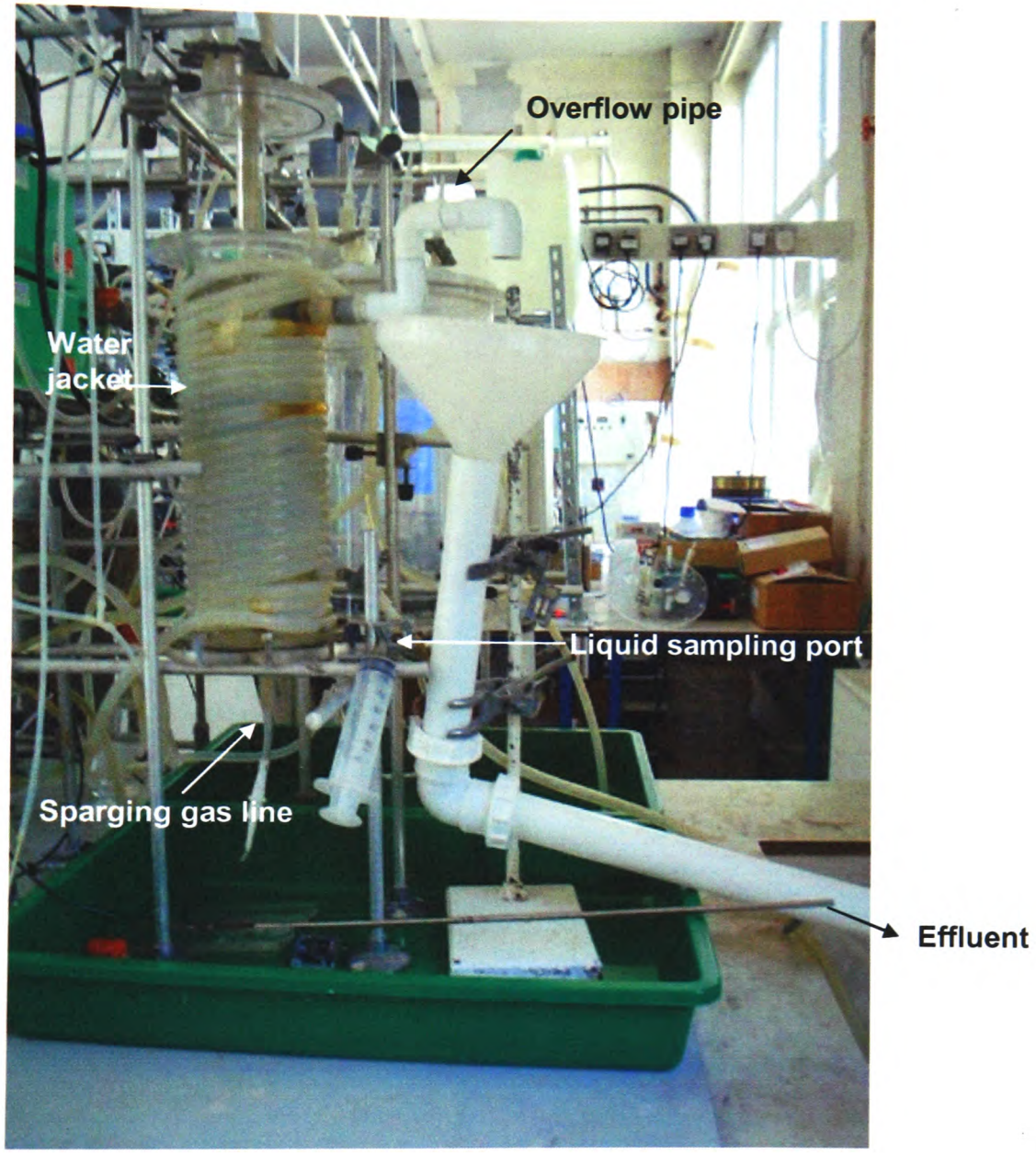


*Figure 9-2. Photo of reactor B during continuous operation*

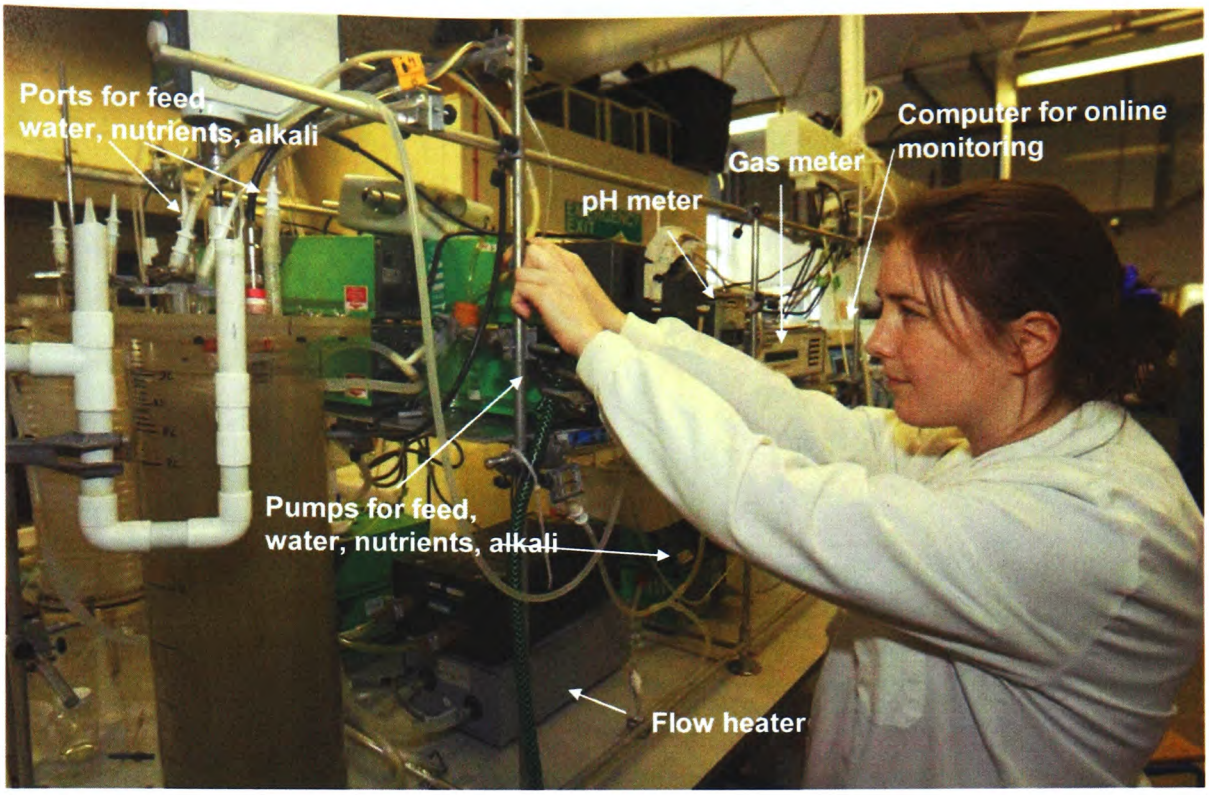
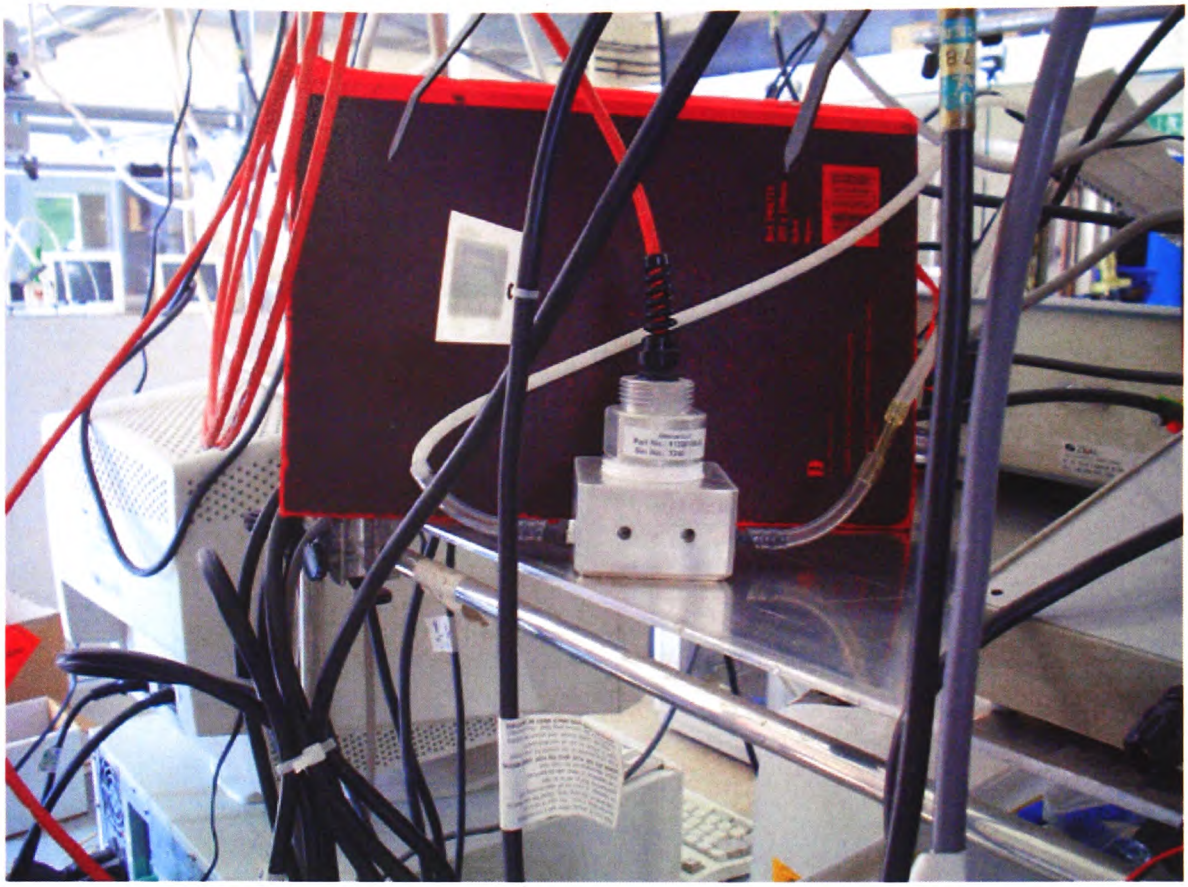


*Figure 9-3. Photo of hydrogen sensor*



PERGAMON

International Journal of Hydrogen Energy 27 (2002) 1339–1347

International Journal of HYDROGEN ENERGY

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# Sustainable fermentative hydrogen production: challenges for process optimisation

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**Abstract**

This paper reviews information from continuous laboratory studies of fermentative hydrogen production useful when considering practical applications of the technology. Data from reactors operating with pure cultures and mixed microflora enriched from natural sources are considered. Inocula have been derived from heat-treated anaerobically digested sludge, activated sludge, aerobic compost and soil, and non-heat-treated aerobically composted activated sludge. Most studies are on soluble defined substrates, and there are few reports of continuous operation on complex substrates with mixed microflora to produce $H_2$. Methanogenesis which consumes $H_2$ may be prevented by operation at short hydraulic retention times (around 8–12 h on simple substrates) and/or pH below 6. Although the reactor technology for anaerobic digestion and biohydrogen production from complex substrates may be similar, there are important microbiological differences, including the need to manage spore germination and oxygen toxicity on start-up and control sporulation in adverse circumstances during reactor operation.


*Keywords:* Hydrogen production; Fermentation

## 1. Introduction

Hydrogen can be produced sustainably by dark, anaerobic bacterial growth on carbohydrate-rich substrates, giving organic fermentation end products, $H_2$ and $CO_2$. Pure cultures known to produce hydrogen from carbohydrates include species of *Enterobacter* [1], *Bacillus* [2] and *Clostridium*. The latter two groups are characterised by the formation of spores in response to unfavourable environmental conditions such as lack of nutrients or rising temperature. Some clostridial species are able to degrade insoluble starch without pretreatment, while some *Enterobacter* sp. studied can degrade soluble starch [3]. The highest $H_2$ yields per mole of hexose have been found for *Clostridium* sp. (1.61–2.36 mol/mol glucose [4]).

*Enterobacter* and *Bacillus* sp. have been used in monocultures. For a technologically feasible process, stable mixed cultures easily obtainable from natural sources able to operate on non-sterile feedstocks are required. Mixed cultures enriched from natural environments are reported to contain mostly clostridia. Such a stable mixed culture isolated from an explosion in a grain silo has been maintained in Japan for a decade (as used by, e.g. Lay et al. [5], Mizuno et al. [6]). A readily available source of hydrogen-producing mixed microflora used by several workers (e.g. [5]) is heat-treated sewage sludge, often anaerobically digested. It is necessary to avoid the presence of organisms utilising $H_2$, particularly methanogens, and this has been achieved in laboratory studies by operating at low pH and /or (for continuous studies) short retention times, since methanogens are more affected by lower pH and are slower growing than fermentative organisms.

Studies in the laboratory have concentrated on pure substrates including glucose, starch and cellulose, often in batch processes. If the inoculum consists of spore-formers, it is possible that growth on pure starch or cellulose may meet

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0360-3199/02/$ 22.00 
PII: S0360-3199(02)00090-3

difficulties not found with complex feedstocks, since spore germination, like sporulation, may require species-specific nutrients [7]. For a viable technology, continuous processes using non-sterile fermentable organic feedstocks are necessary. Complex feedstocks studied, mostly in batch trials, include municipal solid waste [5] and food industry wastes [8].

Process conditions including the inoculum have a significant effect on $H_2$ yield, as they influence the fermentation end products. Fermentations of hexose to acetate or butyrate produce $H_2$ and $CO_2$. Fermentations to propionate or lactate produce no $H_2$. Reduced fermentation end products such as ethanol and other alcohols contain additional H atoms not present in the corresponding acids, so alcohol production gives correspondingly lower $H_2$ yields. It is important therefore to establish bacterial metabolism resulting in acetate and butyrate as end products. The fermentation end products in the effluent from the hydrogen-producing stage need further processing. To obtain maximum hydrogen yield, photofermenting bacteria could in principle be utilised [9]. The fermentation end products are also eminently suitable for anaerobic digestion, yielding methane.

The fermentative process generating $H_2$ has similarities with industrial-scale processes such as the acidogenic stage of anaerobic digestion and acetone–butanol (solvent) production by clostridia. Well-known fermenter technology is used, so reactors are readily available. However, the physiological and physico-chemical conditions under which the microorganisms give optimal $H_2$ yields are not established. Thus the design parameters for the reactors operating continuously on biomass feedstocks are as yet not clearly defined. This paper concentrates on continuous, not batch, results and draws together information from the literature relevant to the design of full-scale processes.

## 2. Feedstock

For sustainable biohydrogen production the feedstock will need to meet certain criteria. These are that the feedstock will be principally carbohydrate, be produced from sustainable resources, be of sufficient concentration that fermentative conversion and energy recovery is energetically favourable, require minimum pretreatment and be of low cost.

Carbohydrates are the preferred organic carbon source for hydrogen-producing fermentations. Glucose (or in principle its isomer hexoses or its polymers starch and cellulose) in biomass gives a maximum yield of $4H_2$ per glucose when acetic acid is the by-product

$$C_6H_{12}O_6 + 2H_2O2 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2. \quad (1)$$

Half of this yield per glucose is obtained with butyrate as the fermentation end product

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2. \quad (2)$$

Glucose and sucrose are the fermentation substrates most studied in the laboratory [10,11]. Ueno et al. [8] operated a laboratory reactor continuously for over 200 days on sugar factory wastewater and obtained a yield of 2.52 mol hydrogen per mol glucose from sugar factory wastewater by a mixed microflora in chemostat culture. Lay [12] using mixed microflora with 0.75% soluble starch obtained 2.14 mol $H_2$/mol hexose.

To date the majority of research has been directed at expensive pure substrates or to a much lesser degree solid waste or wastewaters, however for a truly sustainable process and to meet the demand for renewable energy more sustainable feedstocks will need to be utilised. These could include sugar-containing crops such as sweet sorghum and sugar beet, starch based crops such as corn or wheat, or ligno-cellulosics such as fodder grass and *Miscanthus*.

$H_2$ production from fermentable biomass has the advantage over ethanol production that the microflora is able to use a wider range of cellulosic substrates than the yeasts on which ethanol production is chiefly based, so that feedstock costs should be more competitive. Due to this wider range of substrates utilisable, less feedstock processing should be required than for ethanol production, resulting in greater net energy gain and lower costs. Cellulase-producing clostridia were identified in a mixed $H_2$-producing microflora from forced-aeration sludge compost operating continuously on powdered cellulose at 60°C [13]. However, no $H_2$ producing bacterium has been found to produce both cellulase and hemicellulase. Taguchi et al. [4] demonstrated a pure strain of *Clostridium* could produce $H_2$ effectively in continuous culture at pH 6.0 on xylose, a major product of hemicellulose hydrolysis. The $H_2$ yield at a hydraulic retention time (HRT) around 5 h was 2 mol/mol xylose consumed, and the strain gave similar results when grown on glucose, which was consumed in preference to xylose. Taguchi et al. [14] also report continuous $H_2$ production from the same strain from a cellulose hydrolysate. The carbohydrate-containing feed to the clostridial strain was in each case supplemented with peptone and yeast extract. Yokoi et al. [15] have obtained $H_2$ production in repeatedly fed batch cultures from a defined microbial consortium operating on sweet potato starch residue containing starch, cellulosics and lignin, once used for citric acid fermentation. 0.1% polypeptone was an indispensable nutrient addition, and urea or ammonium salts could not substitute. Ueno et al. [13] operating a continuous reactor thermophilically on powdered cellulose/yeast extract/peptone/mineral salts with a culture enriched from aerobic compost, showed when $NH_4Cl$ replaced peptone as a nitrogen source, $H_2$ yields were halved. These studies show the need for an appropriate complex source of N in media rich in starch, cellulose or hemicellulose products.

To be a sustainable energy production system the process should have a positive energy gain. Hydrogen production studies have used dilute substrates (typically 1% TS, e.g. 10 g/l glucose), but because of the heating requirement for mesophilic operation, net energy production should improve with higher total solids substrate. Therefore a sustainable substrate should be of the highest possible % total

solids, however problems may exist with product inhibition at higher feedstock concentrations. Lay [16] using batch studies on microcrystalline cellulose found an upper limit of feedstock concentration at 25 g cellulose/l, above which specific hydrogen production (as $H_2$ per gVSS of initial inoculum/day) fell. The $H_2$ yield (mol $H_2$/g cellulose added) fell throughout the range (12.5–50 g/l cellulose) studied. However, $H_2$ production at higher TS has been achieved in batch studies on canteen waste/night-soil sludge/sewage treatment plant sludge at around 15% total solids [5], when Lay et al. obtained good hydrogen production for about 24 h after lag periods of up to 2.2 days. We are not aware of any reports of continuous operation on these solid wastes.

Careful consideration will have to be given to the additional inorganic nutrients required by these principally carbohydrate feedstocks for optimal hydrogen production. A comparison of mineral salts media used in laboratory studies shows a varying phosphate and iron content and C:P ratio (Table 1) together with varying amounts of trace elements. Supporting the importance of phosphate as a nutrient, in continuous studies with *C. pasteurianum*, Dabrock et al. [17] found under phosphate limitation, ethanol, butanol and 1,3-propandiol were the major products from glucose. They further report from batch experiments on glucose that iron concentrations of less than 0.56 mg/l are growth limiting for *C. pasteurianum* and cause significant lactic acid production. Although they report iron limitation did not cause a decrease of $H_2$ production in continuous culture, they indicate it resulted in high levels of lactate, as with batch culture. Iron is a component of the hydrogenase enzyme, which generates $H_2$, and iron limitation results in lowered hydrogenase activity. Lee et al. [18] reported from batch studies with mixed cultures on sucrose that low iron concentrations favoured ethanol and butanol production, whilst maximum hydrogen yields were observed when 800 mg $FeCl_2$/l was added to the growth medium. Reduced fermentation end products such as lactate, ethanol and butanol must be avoided in $H_2$ generating cultures to maximise $H_2$ yield, so phosphate and iron must be present at greater than limiting levels. For comparison, the C:P recommended for anaerobic digestion is about 130:1 (corresponding to a COD:P of 350:1 for carbohydrate).

## 3. Inoculum and start-up

The yield of $H_2$ from *Clostridium* species is generally higher than that from the facultatively aerobic *Enterobacter* sp. Yokoi et al. [22] state that *Enterobacter* sp. produce about 1 mol hydrogen per mol hexose, whilst *Clostridium* sp. produce around 2 mol hydrogen per mol hexose. Hence it is advantageous to select for clostridial species in the inoculum. Disadvantages of clostrida which must be allowed for in the process technology are their sensitivity to inhibition by $O_2$, and the specific nutrient and other environmental requirements for spore germination if sporulation occurs in response to unfavourable environmental conditions such as lack of nutrients.

Clostridial spore-formers are selected from natural environments by heat treatment. Pasteurised (two periods of 20 min at 80°C) activated sludge [23] and boiled (15 min) anaerobically digested sludge [12] have both been shown to give successful start-up of continuous laboratory-scale reactors on glucose and starch, respectively. Ueno et al. [8] used as inoculum aerobically composted activated sludge. Although they did not get stable $H_2$ production for 26 days (2 day HRT, pH 6.8), a culture giving stable $H_2$, acetate and butyrate production had by then developed. Thus an inoculum from an aerobic source without pasteurisation can give a successful process after a long start-up, even at a pH and retention time which might give rise to methanogenic populations.

Van Ginkel et al. [24] obtained inoculum for batch $H_2$-producing experiments on sucrose by heat-treating compost, and soil (20 cm deep) from fields on which potatoes or soya beans had been grown. Samples were treated at 104°C for 1 h. A lag period of 50 h occurred before gas production started corresponding to germination, which required the presence of both sucrose and mineral nutrients.

Table 1
Phosphate and iron concentrations in media used for laboratory-scale experiments

| Substrate | Total P (mg/l) | C:P[a] (g/g) | Total Fe (mg/l) | Reference |
|---|---|---|---|---|
| Glucose | 101.51 | 28:1 | 17.24 | [19] |
| Glucose | 44.56 | 90:1 | 1.00 | [6] |
| Glucose | 77.53 | 52:1 | 2.41 | [10] |
| Glucose/glycerol | 310.16 | 26:1 to 260:1 | 9.86 | [17] |
| Cellulose | 148.54 | 34:1 to 135:1 | 0.80 | [16] |
| Sucrose | 44.56 | 90:1 | Varied, optimum 352.73 | [18] |
| Glucose | 22.28 | 360:1 | 5.00 | [20] |
| Beancurd waste | 23.17 | 310:1 | 56.40 | |
| Cellulose | 656.27 | 6:1 | 0.00 | [21] |

[a]C calculated from carbohydrate content as mg/l.

At laboratory scale two methods of limiting methanogenesis are used, either singly or together, operation at a short HRT to wash out methanogens, and operation at low pH to inhibit them. Chen et al. [11], operating on a sucrose-mineral salts medium, started up from non-heat-treated sewage sludge with a gradual decrease of HRT in semi-continuous mode from 20 to 2.5 days. Rather than using low pH, they relied on operating at a HRT down to 6 h at pH 6.7, selecting a culture dominated by *C. pasteurianum*. Optimal $H_2$ production rate was obtained at an 8 h HRT, while below 6 h washout occurred. Using both methods of limiting methanogenesis, Lin and Chang [25], utilising seed from a sewage sludge digester, operated continuous reactors with glucose (20 g COD/l)—mineral salts medium at pH 5.7 or 6.4 and HRTs between 2 days and 6 h. At 6 h HRT at both pHs only approximately 80% of the glucose was degraded, but by contrast at 12 hour HRT 99% of the glucose was degraded and $H_2$ yields around 1.7 mol/mol glucose were obtained, with high butyrate concentrations.

A range of pHs are reported optimum for fermentation of carbohydrates by mixed bacterial cultures. Zoetemeyer et al. [26] operating with glucose at pH 5.7 showed optimum $H_2$ production rate and acetate/butyrate production with negligible propionate at a HRT of 4 h. At pH 6.44 the optimal HRT for $H_2$ production was 7.1 h. Fang and Liu [19] systematically investigated the pH optimum of $H_2$ production from glucose at a 6 h HRT over the range 4.0–7.0 and found the optimum yield at pH 5.5.

If start-up has utilised heat-treated sewage sludges, the resulting sporeforming microflora, if chiefly clostridia, may be unable to consume $O_2$ by aerobic respiration, and so be unable to lower the redox potential and stimulate $H_2$ production. This situation is unlike that in conventional anaerobic digestion, where facultative aerobes will maintain an undetectable oxygen concentration. Clostridia will be inhibited by oxygen present in the liquid medium or introduced from the headspace by mixing. Those working with pure cultures of clostridia, such as Yokoi et al. [22] working with *C. butyricum* are aware of this problem, and these workers demonstrated that the inclusion of a reducing agent in the growth medium to lower the redox potential, or (more cost-effectively) of the facultative $H_2$-producing aerobe *Enterobacter aerogenes* had a protecting effect. With mixed microflora, Ueno et al. [13] flushed the headspace with argon on start-up, although the effect of this was not studied.

Heat-treated sludge inocula will contain sporulated bacteria. Spore germination into a fully active vegetative cell may require specific nutrients (e.g. amino acids). The absolute requirement for 0.1% polypeptone demonstrated by Yokoi et al. [15] for fed-batch cultures of *C. butyricum* and mixed *C. butyricum/E. aerogenes* operating on sweet potato starch residue may be related to the need to break sporulation in this non-continuous feeding mode. Spore germination may require particular environmental conditions, and take an appreciable period. In batch studies with heat-treated sludge inocula a lag of 2 days on the municipal solid waste/sludge mixture [5] or 4 days for microcrystalline cellulose [16] was seen.

The fed-batch method of start-up [15] is not ideal. Cohen et al. [23] demonstrated that a 6 h interruption in feed (glucose and casein hydrolysate) supply could trigger mixed anaerobic spore-formers to sporulate and that sporulation was not reversed by re-supply of glucose after 6 h. Without casein hydrolysate, incomplete sporulation occurred. Thus a constant feed supply during start-up is preferred.

Thus there are important microbiological differences between anaerobic digestion and fermentation of complex substrates with mixed microflora to produce $H_2$. These differences have a practical effect on the process operating conditions.

## 4. Product inhibition

Hydrogen partial pressure in the liquid phase is one of the key factors affecting $H_2$ production. $H_2$ production is a means by which bacteria re-oxidise reduced ferredoxin and hydrogen-carrying coenzymes, and these reactions are less favourable as the $H_2$ concentration in the liquid rises. An analogous situation occurs in fermentation to produce the biofuel ethanol, where ethanol inhibits growth and production rates. Thus a decrease in partial pressure of $H_2$ should give an enhanced $H_2$ yield. We have demonstrated [6] that lowering dissolved $H_2$ by sparging with $N_2$ gave a 68% increase in $H_2$ yield from a reactor operating on an enriched mixed microflora with 10 g/l glucose-mineral salts at pH 6.0, 8.5 h HRT.

Dissolved $H_2$ concentrations may be related to substrate concentration. Lay [16] reported from batch tests that concentrations of microcrystalline cellulose over 25 g/l significantly inhibited hydrogen production, with a maximum hydrogen yield occurring at a cellulose to initial sludge inoculum concentration of 8 g cellulose/g VSS. Dissolved $H_2$ concentrations may also be related to stirring regime; Lay [12] stated an increase in agitation speed of a continuous laboratory-scale reactor mixed by a magnetic stirrer (from 100 to 700 rev/min) more than doubled the daily rate of hydrogen production from starch.

## 5. Metabolic shift

Reduced fermentation end products (e.g. ethanol, butanol, lactate) represent hydrogen that has not been liberated as a gas. Fermentation end products vary within the same bacterium dependent on environmental conditions, and it is necessary to direct metabolism away from alcohols or reduced acids such as lactate and towards volatile fatty acid (VFA) production. In contrast, the conditions favouring acetone–butanol production were well studied in the last century as

solvents were produced commercially fermentatively. The inverse of these conditions is required for $H_2$ production.

*C. pasteurianum* does not grow well at low pH, and is a classic VFA and $H_2$ producer, but its metabolism of glucose can be influenced away from $H_2$ production and towards solvent production by high glucose concentrations (12.5% w/v), by CO (an inhibitor of hydrogenase) and by iron limitation but not by phosphate limitation [17]. *C. acetobutyricum* is a clostridial species best known for its ability to produce solvents (acetone, butanol, ethanol), favoured by pH lower than 5 and phosphate and iron limitation. To give a higher $H_2$ yield, a pH above 5, phosphate and iron above limiting levels and glucose concentrations below 12.5% are the recommendations arising from this pure culture work.

Environmental factors such as pH and HRT may affect the metabolic balance, as does the growth phase in batch culture. Clostridia produce VFA and $H_2$ in the exponential growth phase and rapid alcohol production occurs in late growth phase [12]. Lay et al. [5] in batch trials with a MSW mixture and boiled sewage sludge inoculum showed that $H_2$ and VFA were produced after a 2.2 day lag period, followed on days 3.2 and 4 by propanol/butanol production as the $H_2$ production decreased. Similar alcohol production after the peak of hydrogen/VFA production was found in batch studies using heat-treated sludge inoculum and microcrystalline cellulose [16].

During successful continuous hydrogen production ethanol concentrations are low (e.g. [11]). At a 6 h HRT operating on glucose with an activated sludge inoculum, product distribution was affected by temperature [10]. Butyrate concentrations fell sharply above 30°C, while acetate and propionate concentrations increased. This suggests 30°C may be the optimum temperature for butyrate/$H_2$ production.

## 6. Population shift

The optimum $H_2$ yield should be achieved with acetate as the fermentation end product. In practice, high $H_2$ yields are usually associated with butyrate production, and low yields with the production of propionate, and reduced end products (e.g. alcohols, lactic acid). Clostridia such as *C. butyricum* produce predominantly butyrate. *C. propionicum* types produce mainly propionate, as do other non-sporeformers. Vavilin et al. [28] gave the overall equation for the production of propionate from hexose, showing that this involves the consumption of $H_2$:

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O. \quad (3)$$

Thus the production of propionate should be avoided. Vavilin et al. state that the limiting substrate for butyrate production is glucose, while the limiting substrate for propionate production is $H_2$, and the two groups of organisms producing these end products are in balance in the microbial consortium producing $H_2$. Limiting the amount of propionate-formers by heat treatment of the inoculum may aid in biasing the community towards butyrate production.

Cohen et al. [23] demonstrated that irregular feeding rate (a 2-h daily interruption of supply) to a reactor inoculated with unpasteurised activated sludge strongly selected for non-spore forming propionate-formers (*Selenomonas* sp.). The culture had previously been producing butyrate and $H_2$. A one-off cessation of feed supply for 6 h, or regular feed interruption for 1 h per day gave similar shifts in product formation, thought to be related to a population shift away from butyrate/$H_2$ producing spore formers and towards propionate producing non-spore formers. The semi-continuous feeding mode used by some workers at laboratory scale could thus give poor performance. Also, under continuous operation, it is possible that a short process disturbance may commit the spore-formers irretrievably to sporulation, and the spore-forming population may be then depleted by wash-out if the HRT is short, even though normal feeding is quickly resumed.

Using activated sludge inocula and continuous operation on a glucose-mineral salts medium over a range of HRT from 14 to 3.6 h at pH 5.5 or 6.0, Cohen [29] showed a linear inverse relationship between propionate and butyrate formation, and a linear positive correlation between butyrate and $H_2$ production. There was also a strong linear inverse correlation between redox potential and butyrate production over the range −300 mV (maximum butyrate) and −120 mV (zero butyrate). It was assumed this was due to selection for propionate-producing species as the redox potential rose. It is thus important for maximal $H_2$ yield that the redox potential of the reactor remains near −300 mV.

The complex nature of consortia in $H_2$ producing microflora and the existence of shifts in population is now being demonstrated using genetic techniques. Fang and Lui and Fang et al. [19,27] using gene profiling techniques demonstrated the complexity of a microbial community producing $H_2$ from glucose, and that the microbial community changed with pH in the range studied (pH 4.0–7.0). Horiuchi et al. [30] also studied the effect on organic acid production of changes in operating pH (from 5.0 to 8.0) of continuous reactors inoculated with anaerobic digester sludge and fed with a glucose-yeast extract medium. A switch from butyrate to propionate production as the pH increased was attributed to a change in the dominant microbial population during the transition period of around 120 h, rather than a metabolic pathway change within the same bacterial population, which would be expected to occur more quickly.

## 7. Process technology

Although hydrogen has been sustainably produced in the laboratory both in batch and continuous operation there are as yet no known full-scale plants operating

commercially. The following summarises recommendations based on laboratory-scale continuous studies which should form a basis for scaled-up operation.

### 7.1. Inoculation start-up

The use as seed of heat-treated anaerobically digested sewage sludge in scaled-up reactors could be technically feasible, possibly using steam injection to raise the sludge to boiling point for 15 min. Agricultural soil heat treated to around 100°C for 1 h may also be a suitable inoculum, and the treatment required may be more economic than that of a liquid sludge. However, the mineral matter in the soil inoculum may give handling difficulties, as it will remain in the reactor. Non-heat-treated activated sludge can be used, but as operating conditions (redox, HRT, load) change there is the risk that fermentation products will switch as propionate-formers are selected. Aerobic sources can also be used—an inoculum from forced-aeration sludge compost has been used successfully for thermophilic start-up [13].

After inoculation with heat-treated sporulated seed, specific nutrients and substrates (e.g. glucose, amino acids) must be present for germination, which may take several days. If feeding commences in this interval, wash-out may occur. Careful monitoring and control of the start up process is envisaged as being necessary at full scale.

### 7.2. Process design and operation

From laboratory-scale work on simple substrates, a pH range between 5.5 and 6.7 and HRT of 8–12 h are generally reported to give successful operation, although some workers have used HRTs as low as 4 h and pH below 5.5. Additional alkali is of course required to neutralise the acids when the control pH is closer to neutrality.

Cohen et al. [23], whose main aim was not $H_2$ production, suggested that running an acid digester may be fraught with undesired alterations of product formation (and hence $H_2$ yield) when irregular or low feeding rates are applied. Buffer tanks could be incorporated to even out irregular influent supply rate and concentration, but natural bacterial preacidification will tend to occur in the buffer tank, lowering the yield of $H_2$ in the reactor. The possibility that the microflora may sporulate and wash out if the feed supply is irregular highlights the need for process monitoring and control.

Biomass values in reactors operating on pure soluble carbohydrates are around 1.5–2.5 g/l [11]. Process intensification would suggest it is economic to maintain higher biomass concentrations in the reactor for example by membrane processes, centrifugation, immobilisation or granulation. Kumar and Das [3] working with immobilised *Enterobacter* on naturally occurring solid substrates suggested reactor configurations to avoid gas logging. Immobilisation of *C. butyricum* on porous glass beads has been reported by Yokoi et al. [31]. The development of acidogenic granules was first reported, to our knowledge, by Zoetemeyer et al. [32] at pH 5.6–6.0, with residence times of 1 h at 10 g/l and 6 h at 50 g/l glucose. Fang and Liu [33] reported granule formation of a hydrogen producing acidogenic sludge which was mainly *Clostridium* species. It may therefore be possible to retain the bacteria at full scale in the future, if working with mainly soluble feedstock.

### 7.3. Increasing hydrogen production rate and yield

Hydrogen build-up is inhibiting to the process. The effect of increased $H_2$ partial pressure may limit the use of higher feed strengths. Increased agitator speed may also lower dissolved $H_2$ concentration. For maximum hydrogen yield $H_2$ should be removed as it is produced. Our own research [6] showed that sparging with nitrogen increased $H_2$ yield. We are investigating more technically feasible methods of sparging, e.g. with fuel cell exhaust gas. It should be noted that CO inhibits hydrogenase [17] so the gas chosen for sparging should be free of CO.

A technique which may be applicable to fermentative $H_2$ production was described by Voolapalli and Stuckey [34]. They used a submerged silicone membrane dissolved gas extraction system in a laboratory-scale anaerobic digester, removing $CO_2$ and $H_2$. Problems encountered in removing $H_2$ from the liquid by this method included reduced efficiency due to biofilm build-up. In the anaerobic digester studied by Voolapalli and Stuckey the biofilm was thought to be made up of retained slow-growing methanogens. In a fermentative hydrogen-generating reactor, providing a solid support medium such as the tubing as a basis for attached methanogen growth would be a disadvantage as it would reduce $H_2$ yield; however, methanogenic growth should be prevented by operation at low pH. Our experience suggests acidogenic biofilm growth on this tubing in a $H_2$-producing reactor could cause fouling problems.

Nielsen et al. [35] report another potentially useful method for removing $H_2$ from the gas stream, using a heated palladium–silver membrane reactor. Simulated household waste was fermented for 25 h in a batch laboratory-scale reactor sparged with $N_2$. The off-gas was passed over a membrane with a high selectivity for $H_2$. $H_2S$ stripping must be used, as contamination of the membrane by constituents of the off-gas (e.g. CO) is a potential problem with this technology. Although the membrane effectively reduced $H_2$ in the off-gas, the effect on $H_2$ yield was not studied.

Phosphate limitation may favour solvent production over $H_2$/VFA production, so phosphate addition may be needed with carbohydrate-rich, nutrient-poor feedstocks, and this cost should be included. Ueno et al. [13] have demonstrated with a cellulose influent that use of $NH_4Cl$ rather than peptone halves the $H_2$ yield, so appropriate N-containing nutrients may be needed with N-poor feedstock.

### 7.4. Operating temperature

Temperatures similar to those for mesophilic anaerobic digestion are routinely used, and an optimum may be 30°C. However, thermophilic operation of continuous laboratory reactors has also been reported. This should reduce dissolved $H_2$ concentration but requires a greater energy input which may only be warranted if spare process heat is available on-site.

Thermophilic degradation of powdered cellulose has been observed in continuous culture. The diverse microbial community with efficient $H_2$ production continuously at 60°C has been studied in media including yeast extract, peptone and cellulose powder [13]. Thermophilic continuous operation on glucose had been reported by Zoetemeyer et al. [10] with an optimum temperature of 52°C. At 50°C, increasing the organic load by decreasing the HRT from around 2 to 1.5 h during a 12 day experiment lead to a major decrease in butyrate and increase in ethanol concentration, reversible as the HRT reverted to 32 h. Thus, although shorter retention times may be used thermophilically, the process may be less stable to small changes in HRT/load and need good on-line monitoring and control.

### 7.5. Process monitoring

Redox potential may be a useful on-line monitoring parameter, as a rise in redox potential is associated with a switch from butyrate to propionate production. $O_2$ toxicity may also be significant in start-up with heat-treated inocula lacking a respiratory substrate (e.g. start-up on starch or cellulose) and/or lacking facultative aerobes to remove $O_2$ by respiration. In pH controlled systems the rate of acid production may be followed continuously by monitoring alkali addition. A slower rate of acid production might indicate lower feed strength or microbial washout, possibly due to irregular feeding and sporulation. For soluble feedstock, an on-line measurement of feed strength (TOC, COD, etc.) could distinguish between these possibilities.

The situation with a $H_2$ fermenting reactor is unlike that in a wastewater treatment process. In, for example, anaerobic digestion, incoming wastewater must be treated and only a small proportion can be diverted as a control measure. However, assuming sufficient $H_2$ is stored to meet the requirements of energy use, the feed rate to a $H_2$ fermenting reactor can be a flexible control variable in response to microbiological changes.

### 7.6. Product use

Fig. 1 shows a possible scheme for local $H_2$ production by fermentation of biomass. From the first stage reactor hydrogen is formed and the by-product fermentation end products (chiefly acetic and butyric acids) pass to a second methanogenic stage. Hydrogen can be used alone in an internal combustion engine or a fuel cell, it can be combined with the methane from the second stage and used in an engine, or methane can be used alone either in an engine or in a reforming fuel cell. For processes growing fermentable energy crops and generating hydrogen locally, the liquor resulting from anaerobic digestion, retaining the N and P content, could be used with full traceability as a fertiliser for the crop.

## 8. Conclusions

A continuous scaled-up process for sustainable fermentative $H_2$ production has not yet been reported in the literature. Such a process may be seeded with sewage sludge from anaerobic or aerobic processes, with heat treatment of the inoculum giving faster start-up. Information from laboratory-scale work on continuous processes suggests operating at 30°C, at a pH around 5.5, and HRT approximately 8–12 h for simple substrates. The process

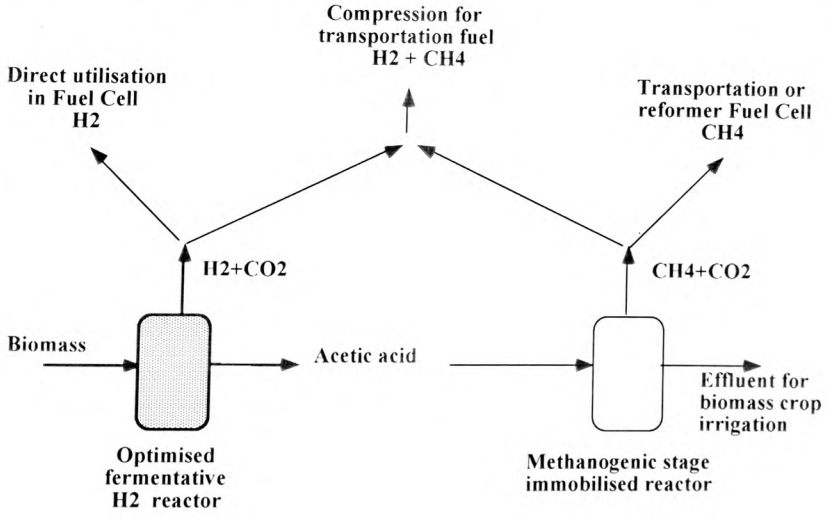


Fig. 1. Scheme for holistic biological $H_2$ production from energy crops.

using carbohydrate-rich feedstock may be further optimised by adding sufficient phosphate, a complex nitrogen source and the introduction of technologies to lower dissolved $H_2$. On-line control, e.g. by monitoring gas flow, gas composition and liquid redox potential may be required to prevent deviations from acetate/butyrate/$H_2$ producing metabolism and wash-out of spore-formers following process disturbances.

## Acknowledgements

This work was partly funded through EPSRC Grant number GR/R22520/01.

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# Continuous Fermentative Hydrogen Production from a Wheat Starch Co-Product by Mixed Microflora

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*Received 18 January 2003; accepted 18 June 2003*

*DOI: 10.1002/bit.10785*

**Abstract:** For the transition to the hydrogen economy, hydrogen must be produced sustainably, e.g., by the fermentation of agricultural material. Continuous fermentative production of hydrogen from an insoluble substrate in nonsterile conditions is yet to be reported. In this study hydrogen production using mixed microflora from heat-treated digested sewage sludge in nonsterile conditions from a particulate co-product of the wheat flour industry (7.5 g $L^{-1}$ total hexose) at 18- and 12-hour hydraulic retention times, pH 4.5 and 5.2, 30°C and 35°C was examined. In continuous operation, hydrogen yields of approximately 1.3 moles hydrogen/mole hexose consumed were obtained, but decreased if acetate or propionate levels rose, indicating metabolism shifted towards hydrogen consumption by homoacetogenesis or propionate producers. These shifts occurred both at pH 4.5 and 5.2. Sparging the reactor with nitrogen to reduce hydrogen in the off-gas from 50% to 7% gave stable operation with a hydrogen yield of 1.9 moles hydrogen / mole hexose consumed over an 18-day period. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **XX**: 000–000, 2003.

**Keywords:** sustainable hydrogen production; fermentation

## INTRODUCTION

Hydrogen, an entirely carbon-free fuel with a high combustion enthalpy (185 kJ $L^{-1}$) is considered a feasible alternative to fossil fuels, with the technology for hydrogen as a transport fuel already well established. Hydrogen is currently produced in large amounts by the chemical industry e.g by steam reforming of fossil fuels. For the hydrogen economy hydrogen must be produced sustainably, for example, from water through electrolysis powered by renewable energy, photosynthetically, or by gasification or pyrolysis of biomass. It should also be possible to develop a cost-effective and reliable technology to produce hydrogen directly from renewable biomass or organic waste products by anaerobic fermentation. This process is directly related to the acidogenic first stage of anaerobic digestion, extensively investigated for simple sugars by Cohen et al. (1979; 1985) and Zoetemeyer et al. (1982a; 1982b). Carbohydrate-rich substrates can be directly fermented to hydrogen, carbon dioxide, butyrate, and acetate as shown in Eqs. (1) and (2), with acetate fermentation giving the highest theoretical yield of hydrogen (4 mol/mol hexose):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

However, continuous studies using glucose as substrate and clostridia-type microflora typically report a mixed acid fermentation with butyrate in excess of acetate (Chen et al., 2001; Fang and Lui, 2002; Mizuno et al., 2000).

Research in dark fermentative hydrogen production has often focused on batch studies and pure cultures of bacteria such as the metabolically diverse spore-forming clostridia with species which can degrade mono- and disaccharides, cellulose, and starch (Rogers and Gottschalk, 1993). Yokoi et al. (2001), for example, report yields of 2.4 mol hydrogen $mol^{-1}$ glucose with a pure culture of *Clostridium butyricum* in fed-batch experiments. Lay (2001) obtained a hydrogen yield around 0.6 mol/mol hexose in batch experiments with microcrystalline cellulose with clostridial-type mixed microflora. However, rather than using batch studies and pure cultures, continuous operation with an enriched mixed microflora maximizes reactor productivity, requires no energy for sterilization, and can use technology adapted from the anaerobic digestion process,

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Contract grant sponsor: UK EPSRC
Contract grant number: GR/R22520/01

already well established on an industrial scale. Hydrogen-producing mixed microflora can be selected from sewage sludge, for example, a predominantly clostridial microflora producing hydrogen from glucose was enriched by operation for 30 days at a low pH and short hydraulic retention time (HRT) (Fang et al. 2002a). For more rapid start-up, an inoculum presumably enriched in clostridia can be obtained by heat treatment of sewage sludge or soil (Hawkes et al., 2002). Methanogenesis can be prevented by operation at low pH and short HRT, but when using a mixed microflora there is the potential for other competing reactions that consume hydrogen. These include homoacetogenesis [Eq. (3)] performed by bacteria including some clostridia and propionic acid production [Eq. (4)] by bacteria other than clostridia.

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (3)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (4)$$

The operating conditions suppressing these reactions are not well understood.

Continuous fermentative hydrogen production has been reported from simple carbohydrates such as glucose or sucrose with mixed microflora at 6–8 h HRT (Chen et al., 2001; Fang and Liu, 2002, Mizuno et al., 2000). Ueno et al. (1996) obtained a yield of 2.52 mol hydrogen $mol^{-1}$ hexose at 60°C on sugar factory waste water and Fang et al. (2002b) demonstrated a mixed microflora from sewage sludge at 26°C and 6 h HRT could form granules containing a range of *Clostridium* species giving a hydrogen yield of 2.25 mol $mol^{-1}$ hexose. However, most low-cost agricultural and organic waste products are complex substrates and information on their conversion to hydrogen in continuous processes, whether by defined cultures or mixed microflora, is very limited.

Hydrogen production from sweet potato starch residue with the addition of corn steep liquor has been demonstrated (Yokoi et al., 2002) using a defined culture of *Clostridium butyricum* and *Enterobacter aerogenes* in a daily fed-batch culture over 12 days. Corn steep liquor replaced the polypeptone essential for hydrogen production in an earlier experiment (Yokoi et al., 2001). To our knowledge continuous fermentative hydrogen production from complex carbohydrates by mixed microflora has been reported in only two studies. Ren et al. (1995, 1997) obtained continuous hydrogen production by a mixed culture from molasses. Corn starch was also used, but no data on hydrogen production were given. Lay (2000) reported continuous hydrogen production by a mixed microflora with clostridial characteristics from a soluble starch about which no information was given. From his results a hydrogen yield of 1 mol/mol hexose can be calculated from the enrichment culture at pH 4.5 and 22 h HRT. A factorial-design experiment using this culture as inoculum for experiments of around 6 days duration gave optimal operating conditions for the uncharacterized soluble starch used of pH 5.2 and 17 h HRT with a hydrogen yield which can be calculated to be 2.4 mol/mol. However, as pointed out by Fang et al. (2002b) the figure given by Lay (2000) of 1.29 L hydrogen $g^{-1}$ starch-COD represents a yield (10 mol $mol^{-1}$) substantially higher than the theoretical maximum even if all starch was converted to acetate.

There is thus no information on hydrogen yields obtainable in continuous operation using mixed microflora from any starch from an industrial source, although the opportunities for hydrogen generation from industrial co-products of the starch food industry are immense. Using an insoluble co-product of the wheat starch food industry, start-up with a mixed microflora from heat treated anaerobically digested sewage sludge and the ability of the conditions recommended by Lay (2000) and sparging (Mizuno et al., 2000) to suppress hydrogen-consuming acetogenesis and propionate production was investigated in this study.

## MATERIALS AND METHODS

### Growth Medium

The feed for all experiments contained cold-swelling wheat starch (DEFINOL BWH; Crespel&Deiters GmbH&Co. KG, Ippenbueren, Germany), blended with tap water in a homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) to 80 g $L^{-1}$ and diluted to 10 g $L^{-1}$ at the entry point to the reactor. The starch powder had a hexose content of 728 ± 31 g $kg^{-1}$, 940 g/kg total solids and 960 g/kg COD at delivery. Data on composition as provided by the supplier are shown in Table I.

Mineral nutrients were added in the following concentration at the point of entry to the reactor (modified from Fang and Liu, 2002): $NH_4Cl$ 2600mg, $K_2HPO_4$ 250 mg, $KH_2PO_4$ 250mg, $MgCl_2 \cdot 6H_2O$ 320mg, $FeSO_4 \cdot 7H_2O$ 86mg, $CoCl_2 \cdot 6H_2O$ 15 mg, $MnCl_2 \cdot 4H_2O$ 15 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 14 mg, $Na_2B_4O_7 \cdot 10H_2O$ 12 mg, $NiCl_2 \cdot 6H_2O$ 49 mg, $ZnCl_2$ 23 mg, $CuCl_2 \cdot 2H_2O$ 10 mg and $CaCl_2 \cdot 2H_2O$ 66 mg. The solution was stored as a 13-fold concentrate acidified to pH < 2. In experiments 2, 4 and 5, 1 g $L^{-1}$ pancreatically digested peptone and 1 g $L^{-1}$ glucose (anhydrous D-glucose; Fisher Scientific UK, Loughborough, UK) were added to the reactor influent. Feed and organic nutrients were stored at 4°C.

**Table I.** Properties of Definol BWH starch.

| | |
|---|---|
| Moisture Content | Maximum 10% |
| Protein content | Max. 5.0% |
| Starch content | Min. 75.0% |
| Ash content | Max. 0.8% |
| pH value | Min. 4.0 |
| Particles >315μm | Max. 0.2% |
| Water absorption | Min 1: 6 |

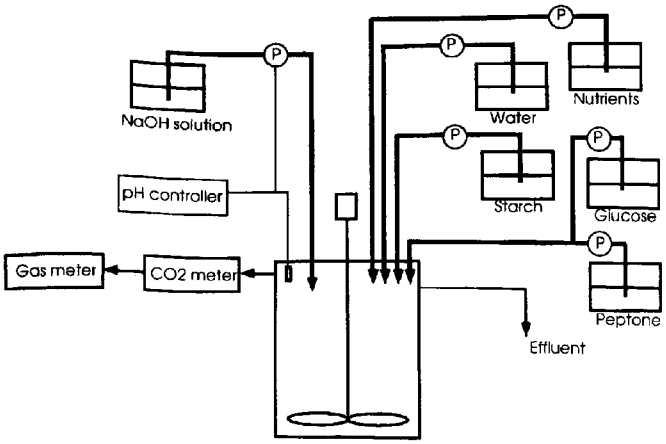


**Figure 1.** Anaerobic continuously stirred tank reactor (CSTR).

## Reactor

Two anaerobic CSTR reactors with working volumes of 9.5 and 2.3 L were used. A schematic of the experimental apparatus is given in Figure 1. Each reactor was stirred with an adjustable stirrer (Heidolph Instruments, Schwabach, Germany) at 100 rpm. Temperature was controlled by a Grant flow heater (Cambridge, UK) to 30 or 35°C through a water jacket. The pH value was kept constant by automatic titration with a Watson Marlow (Falmouth, UK) 505U/RL pump connected to an ABB KentTaylor (Cambridge, UK) or Mettler Toledo (Urdorf, Switzerland) pH controller, using 1*M* NaOH. All ingredients were pumped into the reactor by Watson Marlow 505U/RL or 505S pumps. The liquid level in the reactor was controlled by the position of a U-bend overflow pipe. For sparging a shortened sinter-stick attached to the center of the 2.3-L reactor base was connected to a GC grade nitrogen cylinder from Messer (Reigate, UK). The sparging rate was monitored with a volumetric flow meter from Cole Parmer (Vernon Hills, IL).

Gas production, carbon dioxide content of the produced gas, and culture pH were monitored continuously. Data was logged every 5 min (as the average of data recorded every 30 s) to a Viglen Pentium III computer fitted with a Labview data acquisition system. Gas production was measured with a volumetric flow meter from Agilent (Wilmington, USA) or an Alexander Wright low flow gas meter LM300 operating on a principle developed in this laboratory (Guwy et al., 1995). The percentage carbon dioxide in the produced gas was measured with a meter fitted with an infra-red gas card from Edinburgh Sensors Ltd (Livingstone, UK).

**Table II.** Summary of operating conditions for experiments 1–5.

| Experiment | pH | Temp (°C) | HRT [h] | Duration of batch start-up (h) | Duration of continuous operation (d) |
|---|---|---|---|---|---|
| 1 | 4.5 | 35 | Batch | 55 | - |
| 2 | 5.2 | 30 | 18 | 40 | 9 |
| 3 | 4.5 | 35 | 18 | 40 | 9 |
| 4 | 5.2 | 30 | 18 & 12 | 40 | 21 |
| 5[a] | 5.2 | 30 | 15 | 43 | 18 |

[a] Sparged with nitrogen.

## Start-up, Batch, and Continuous Operation

Anaerobic digester sludge (Cog Moors sewage treatment works, Cardiff, UK) sieved through a 1.18-mm mesh was used for inoculum and stored in a sealed container at room temperature for up to 14 weeks. Sludge to give 3 g $L^{-1}$ TS in the reactor was boiled for 15 min and added to the reactor containing growth medium. The pH in the range 4–7 was left to decrease naturally to the required value, or be increased by the on-line control. Continuous operation was started once significant hydrogen production had occurred.

Nineteen batch experiments were undertaken, of which 14 were converted to continuous operation. Of these, 5 typical and representative experiments are described here. Operating parameters are summarized in Table II.

During experiment 4 the retention time was shortened to 12 h on day 17. In experiments 1–4 the gas in the reactor headspace was self-generated, while for experiment 5 the reactor was sparged with nitrogen at a rate of 59.1 ± 2.8 mL $min^{-1}$, starting as soon as the reactor was filled and continuing for the duration of the experiment.

## Off-Line Analysis

Gas samples were taken from the gas tube between the reactor headspace and the carbon dioxide meter. Fluid samples were taken from a port near the bottom of the reactor. Hydrogen, carbon dioxide, and methane content of the produced gas was determined daily off-line by the method of Mizuno et al. (2000). VFAs were determined daily off-line by the method of Cruwys et al. (2002). Solvents were determined daily by gas chromatography (Varian 3400cx gas chromatograph, FID detector, 2 m carbowax column filled with Carbopack 80/120, injection port and FID temperatures 120°C and 220°C respectively, column temperature increased from 65 to 200°C at

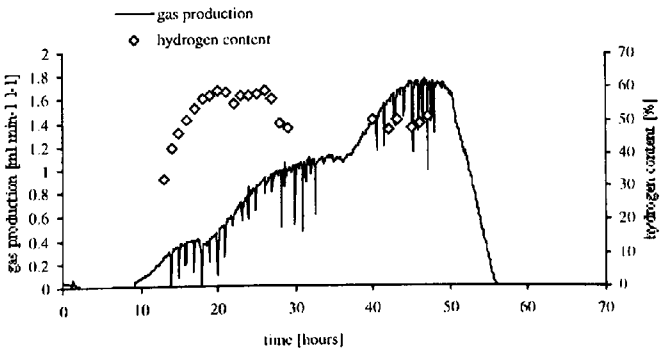


**Figure 2.** Hydrogen production during experiment 1, a typical batch start-up, pH 4.5, 35°C.

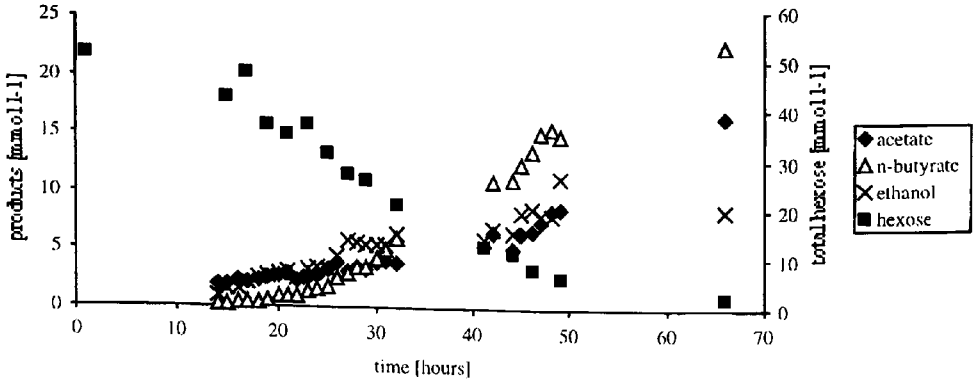


**Figure 3.** Total hexose and product concentrations during experiment 1.

4°C $min^{-1}$). Total solids were determined by weight difference before and after drying following the standard method (APHA, 1989). Glucose and total hexose were analyzed colorimetrically after enzymatic digestion, using the starch assay from Megazyme International Ireland Ltd. (Bray), slightly modified to allow analysis of liquid samples by using 1 mL sample instead of 100 mg solids. For the maize starch standard supplied 10 mg starch was added to 1 mL of deionized water.

## RESULTS AND DISCUSSION

### Batch Start-Up

Hydrogen was produced in each of the 19 batch start-ups at pH 4.5, 5.2, and 6.0. Data from a typical batch start-up are shown in Figures 2 and 3. The biogas consisted of hydrogen and carbon dioxide only. The yield for experiment 1 was 1.44 mol hydrogen $mol^{-1}$ hexose consumed. During the batch start-up of experiment 3 the hydrogen yield was 1.51 mol $H_2$ $mol^{-1}$ hexose consumed, calculated from the cumulative gas production and the hydrogen composition 40 h after start-up, when feeding commenced. For experiment 4 during batch start-up before the start of feeding continuously at 40 h the yield was 1.73 mol hydrogen $mol^{-1}$ hexose consumed. There was thus no difficulty in obtaining a hydrogen yield between 1.4 and 1.7 mol hydrogen $mol^{-1}$ hexose consumed during batch experiments with heat-treated anaerobically digested sewage sludge.

Butyrate, accumulating to 21 mmol $L^{-1}$ (1944 mg $L^{-1}$) in experiment 1 (Fig. 3), was the main VFA produced in all batch start-ups, accompanied by acetate and ethanol production. A final concentration of 16.2 mmol $L^{-1}$ (970 mg $L^{-1}$) acetate and 8.3 mmol $L^{-1}$ (382 mg $L^{-1}$) ethanol was produced in experiment 1. Glucose levels were never above 2.3 mmol $L^{-1}$ (0.4 g $L^{-1}$). In none of the batch start-ups was production of propionate, acetone, or butanol observed.

### Continuous Hydrogen Production Under Self-Generated Gas Atmosphere

The dominant metabolism in a mixed acidogenic culture is reported to depend strongly on culture pH (Lay, 2000). The literature generally points towards pH 5 to 6 for maximum hydrogen yields (Lay, 2000; Fang and Liu, 2002, Zoetmeyer et al., 1982b). However, Ren et al. (1997) state that optimum hydrogen production would occur at pH less than 5. The most commonly reported temperature range for mesophilic continuous $H_2$ production is 35 to 37°C (Lay, 2000; Lin and Chang, 1999; Mizuno et al., 2000), but Zoetemeyer et al. (1982) report from an acidogenic reactor that butyrate production was weakest at 35°C in the mesophilic temperature range. In the work reported here continuous operation under a self-generated gas atmosphere was investigated at pH 4.5 and 5.2. Three representative experiments are discussed. Data from experiment 2 at pH 5.2 are shown in Figures 4 and 5. During the first 8 days of continuous operation more butyrate (17–26 mmol $L^{-1}$ or 1510 to 2250 mg $L^{-1}$) than

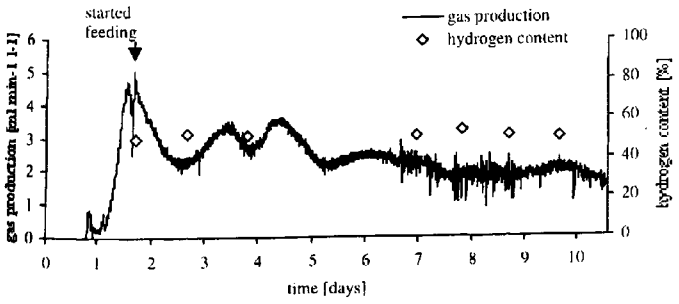


**Figure 4.** Hydrogen production during experiment 2, pH 5.2, 30°C and 18h HRT.

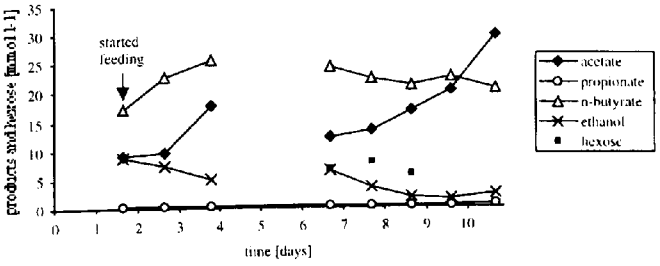


**Figure 5.** Total hexose and product concentrations during experiment 2.

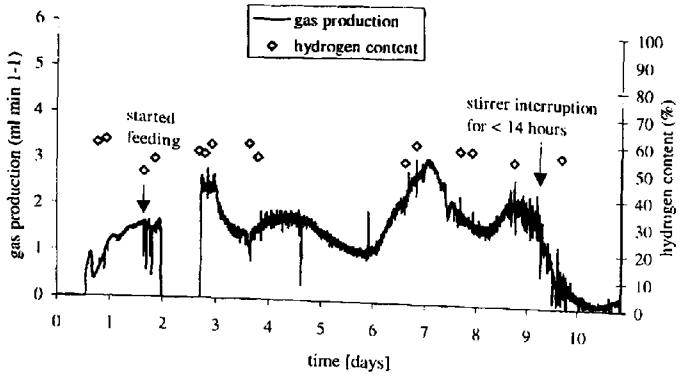


**Figure 6.** Hydrogen production during experiment 3, pH 4.5, 35°C and 18 h HRT.

acetate (9.2 to 20.5 mmol $L^{-1}$ or 550 to 1230 mg $L^{-1}$) was produced. Ethanol was mainly produced during batch start-up (Fig. 5), reaching a maximum concentration of 8.7 mmol $L^{-1}$ (400 mg $L^{-1}$), while during continuous operation the concentration gradually decreased to below 2 mmol $L^{-1}$ (100 mg $L^{-1}$). No propionate, acetone, or butanol was detected. The average hydrogen yield was 1.28 mol $mol^{-1}$ hexose converted during days 7 to 9, when gas production was not oscillating. The produced gas consisted entirely of carbon dioxide and hydrogen, with a hydrogen content of 45–55%. No methane was detected. During days 7 to 11 the acetate concentration more than doubled (from 13.5 mmol $L^{-1}$ to 38.5). From Eq. (1) it would be expected that hydrogen yield would increase with acetate production, but this was not observed. Hydrogen and butyrate production varied little, with an average yield of 1.06 ± 0.12 mol hydrogen $mol^{-1}$ hexose added and butyrate concentration between 24.4 and 20.6 mmol $L^{-1}$. It is possible the metabolism shown in Eq. (3) was developing, either due to a metabolic shift of which the clostridia are capable (Rogers and Gottschalk, 1993) or the development of a population of non-clostridial homoacetogens.

In comparison to experiment 2 hydrogen production during the majority of the other 12 continuous operation experiments with self-generated gas atmosphere at pH 4.5 or 5.2, 30 or 35°C and 18 or 12 h HRT, lasting between 4 and 23 days, was more short-lived and variable over time with lower overall hydrogen yields. Data for experiment 3 at pH 4.5, an example of washout of the culture, are shown in Figures 6 and 7. During the first 7 days of continuous operation a hydrogen-producing pattern similar to that in experiment 2 was observed. Butyrate, acetate, and ethanol were produced in concentrations in the range observed in experiment 2, and there was also no propionate, acetone, or butanol detected. Gas with a hydrogen content of around 50% was produced in an oscillating pattern as in experiment 2, giving an average yield of 1.26 mol hydrogen per mol hexose converted during days 4 to 9. However, washout of all starch fermenting organisms after day 9 was indicated by the fall in gas and acid production in combination with rising residual starch (total hexose) levels, showing the culture was susceptible to disturbances. The washout was most likely caused by failure of the stirrer for less than 14 h on day 9, which will have caused a decrease in pH, starvation, and high hydrogen partial pressure in the bulk of the reactor. A consequence could be sporulation of clostridia (Labbe and Shih, 1997). Since on resumption of favorable growth conditions after a disturbance a lag period may occur as during start-up (see Fig. 2) during which no glucose is available from starch degradation, glucose was added in experiments 2 and 4 in an attempt to improve reactor resilience.

Data for Experiment 4 are summarized in Table III as an example of a series of changes in metabolism during continuous operation. Hydrogen production commenced as in experiments 2 and 3. On day 3 the daily hydrogen yield was 1.25 mol per mol hexose converted and butyrate was the main product with a concentration of 25.6 mmol $L^{-1}$ (2250 mg $L^{-1}$). Signs of the presence of homoacetogens appeared after day 6, when the acetate and butyrate concentrations both increased steeply, from 20.8 mmol $L^{-1}$ (1250 mg $L^{-1}$) to 46.6 mmol $L^{-1}$ (2800 mg $L^{-1}$) and from 18.4 $mmol^{-1}$ (1620 mg $L^{-1}$) to 33.3 mmol $L^{-1}$ (2930 mg $L^{-1}$) respectively, while a corresponding increase in hydrogen yield was not observed. During days 10 and 12 propionate production began, reaching concentrations of 10.5 mmol $L^{-1}$ (777 mg $L^{-1}$ on day 16), which was presumably the reason for the observed drop in average daily hydrogen yields to between 0.5 and 0.8 mol hydrogen $mol^{-1}$ hexose converted.

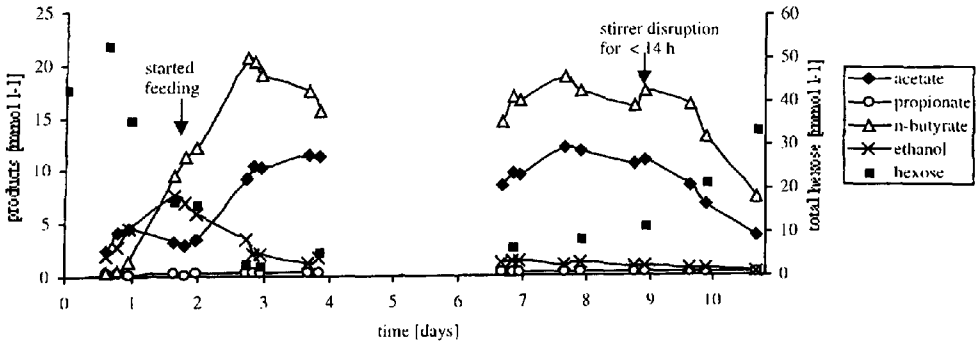


**Figure 7.** Total hexose and product concentrations during experiment 3.

**Table III.** Fermentation end products during experiment 4—pH 5.2, 30°C, 18 and 12 h HRT.

| HRT (h) | Day | Products and Residual Hexose [mmol $L^{-1}$] | | | | | Gas Production ($1L^{-1}$ $d^{-1}$) | H2 (%) | Daily Yield (mol $mol^{-1}$) | Butyrate/ Acetate Ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Acetate | Propionate | *n*-Butyrate | Ethanol | Hexose | | | | |
| Start-up | 1 | | | | | | 0.03 | 49.2 | 0.01 | |
| 18 | 2 | 15.37 | 0.28 | 26.57 | 6.53 | 1.04 | 2.53 | | | 1.73 |
| | 3 | 16 | 0.25 | 25.58 | 5.4 | 1.06 | 3.56 | 48.6 | 1.25 | 1.6 |
| | 4 | | | | | | 3.08 | | | |
| | 5 | | | | | | 1.9 | | | |
| | 6 | 20.84 | 0.25 | 18.42 | 1.9 | 1.06 | 1.32 | 50.3 | 0.48 | 0.88 |
| | 7 | 25.86 | 0.26 | 25.26 | 1.8 | 2.17 | 2.7 | 49.3 | 0.99 | 0.98 |
| | 8 | 26.23 | 0.25 | 22.86 | 1.85 | 1.53 | 2.72 | 50.1 | 0.99 | 0.87 |
| | 9 | 30.87 | 0.36 | 26.18 | 1.75 | 1.58 | 2.3 | 54.8 | 0.93 | 0.85 |
| | 10 | 46.61 | 0.26 | 33.32 | 2.04 | 1.93 | 2.41 | 31.3 | 0.56 | 0.71 |
| | 11 | | | | | | 1.13 | | | |
| | 12 | | | | | | 2.21 | | | |
| | 13 | 40.04 | 5.75 | 19.55 | 1.55 | 2.39 | 2.48 | 43.3 | 0.80 | 0.49 |
| | 14 | 38.5 | 9.43 | 17.76 | 1.06 | 4.03 | 1.97 | 44.3 | 0.68 | 0.46 |
| | 15 | 38.48 | 10.41 | 16.4 | 0.94 | 3.76 | 1.46 | 47.5 | 0.54 | 0.43 |
| | 16 | 34.62 | 10.46 | 13.97 | 1.05 | 3.7 | 1.25 | 52 | 0.50 | 0.4 |
| up 12 | 17 | 27.62 | 7.03 | 15.12 | 1.05 | 4.72 | 1.79 | 53.2 | 0.50 | 0.55 |
| | 18 | | | | | | 2.65 | | | |
| | 19 | | | | | | 2.79 | | | |
| | 20 | 27.82 | 5.89 | 15.64 | 1.23 | 3.54 | 2.36 | 50 | 0.60 | 0.56 |
| | 21 | | | | | | 2.63 | 44.1 | | |
| | 22 | 28.72 | 7.89 | 16.77 | 1.3 | 4.54 | 3.16 | 50.3 | 0.83 | 0.58 |
| | 23 | 28.61 | 6.72 | 15.34 | 1.37 | 3.41 | 3.35 | | | 0.54 |

Cohen et al. (1985) reported from an acidogenic reactor operating on glucose with mixed microflora that the propionic and butyric acid producing metabolisms belong to different species. Butyric acid producers were mainly *Clostridia*, while propionic acid producers were identified as mainly *Selenomonas* sp. On day 17 the hydraulic retention time was decreased from 18 to 12 h to investigate if this would disadvantage hydrogen consumers. Cha and Noike (1997) in chemostat experiments with a mixed acidogenic culture operating on an uncharacterized starch observed propionic acid production at 24 and 48 h but not at 12 h HRT. Here, the shorter retention time led to a reduction in propionate production to 70% of the levels immediately before the change in HRT (Table III) and an increase in hydrogen yield from 0.50 during days 15 to 17 to 0.68 mol hydrogen $mol^{-1}$ hexose converted during days 20 to 23. However, butyrate production was not increased. Similar observations were made in a repeat experiment (data not shown).

Of the continuous experiments not described here, two showed dominant hydrogen and butyrate production throughout. In the remaining experiments hydrogen production was dominant at the beginning of continuous operation, but propionate and/or acetate levels increased while hydrogen yields did not. This suggests that propionate producers and/or homoacetogens began to co-exist with the hydrogen producers in the mixed culture during continuous operation, causing highly variable hydrogen yields as shown in experiment 4.

Observations and theoretical considerations reported in the literature suggest that homoacetogenesis and propionic acid production would occur predominantly at pH 5 or above. Ren et al. (1997), for example, state that propionic acid production was dominant between pH 5 and 6 in continuous hydrogen production with a mixed culture, and thus recommend operation at pH 4.5. A strong presence of propionic acid bacteria at pH 6 was also observed by Cohen et al. (1985). Rogers and Gottschalk (1993) state that homoacetogenesis is not likely to occur at pH 5 or below, because acetic acid functions as an uncoupler. Homoacetogenesis is presumed to occur at pH 5.2 in experiments 2 and 4. It could thus be an advantage to operate at a pH below 5, since this also involves the addition of less alkali for pH control, incurring less cost.

## Continuous Hydrogen Production With Nitrogen Sparging

Biological hydrogen production is inhibited as the partial pressure of hydrogen rises (Ruzicka, 1996). Experiments by Mizuno et al. (2000) showed that hydrogen yields could be increased by 68% for continuous mixed cultures on glucose by sparging with nitrogen. Also, a lower hydrogen partial pressure may suppress hydrogen consuming reactions such as homoacetogenesis or propionate production. A microorganism described by Zinder (1994) was observed at 60°C to grow acetogenically on hydrogen and carbon dioxide at hydrogen partial pressures greater

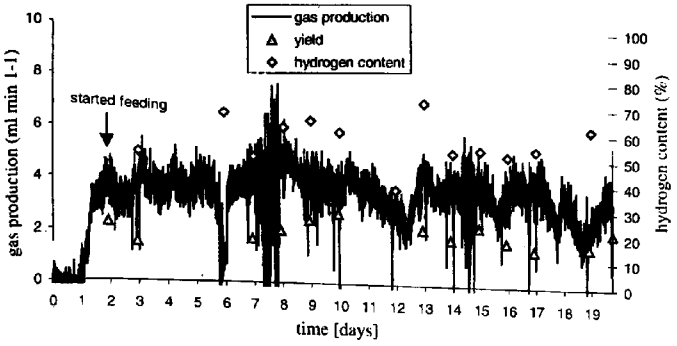


**Figure 8.** Hydrogen production during experiment 5, pH 5.2, 15h HRT, 30°C, nitrogen sparging.

than 500 Pa, while it could oxidize acetate to hydrogen and carbon dioxide at hydrogen partial pressures below 40 Pa. In experiment 5 the culture was continuously sparged with nitrogen to investigate the effect of decreased hydrogen partial pressure on product distribution. The batch hydrogen yield for this experiment was 1.53 mol $mol^{-1}$ hexose consumed. Hydrogen production did not oscillate and was less variable than in experiments without sparging (Fig. 8). Gas production as shown in Figure 8 was calculated as the difference between total gas flow measured exiting the reactor and the flow rate of sparging gas entering the reactor. The hydrogen content of the produced gas was estimated from the calculated gas production and the hydrogen content of the total exiting gas flow, which was on average 7% during days 3 to 19. The average gas production rate after 3 HRT of continuous feeding was 3.6 ± 1.1 mL $min^{-1}$ $L^{-1}$, containing approximately 57% hydrogen, with average sparging rate of 58.1 ± 2.0 mL $min^{-1}$ and average total gas flow of 66.3 ± 3.0 mL $min^{-1}$. The average yield during days 5 to 20 was 1.87 mol hydrogen $mol^{-1}$ hexose converted or 1.68 mol hydrogen $mol^{-1}$ hexose added. During batch start-up and the first 16 days of continuous operation butyrate was the main product with concentrations of 19 to 28 mmol $L^{-1}$ (1670 to 2460 mg $L^{-1}$) (Fig. 9). Acetate concentrations increased gradually during continuous operation from 12 mmol $L^{-1}$ (720 mg $L^{-1}$) on day 3 to 17 mmol $L^{-1}$ (1020 mg $L^{-1}$) on day 19. Propionate production was present from day 12 but did not increase to concentrations over 2 mmol $L^{-1}$ (150 mg $L^{-1}$).

This shows that although a gradual decrease in butyrate production and increase in acetate and propionate production could be observed despite sparging with nitrogen, hydrogen production is less variable and occurs with higher yields than in experiments with self-generated gas atmosphere. Hydrogen yields of 1.87 mol $mol^{-1}$ hexose converted were recorded for 18 days of continuous operation with nitrogen sparging. The change of metabolism is considerably slower and could possibly be suppressed completely by technically improved sparging procedures. Although sparging results in a significant dilution of the product gas, new gas separation systems utilizing polymeric membranes and active membranes technology can separate hydrogen from other gases such as nitrogen, methane, and carbon dioxide at ambient temperatures and pressures (Teplyakov et al., 2002).

## CONCLUSIONS

This work shows that continuous fermentative hydrogen production from particulate wheat starch by a mixed culture is possible. Start-up from heat-treated anaerobically digested sewage sludge by batch operation under a self-generated gas atmosphere took 22–40 hours at both pH 4.5 and 5.2 with a yield reproducibly between 1.4–1.7 mol hydrogen $mol^{-1}$ hexose consumed. Following batch start-up, continuous production of hydrogen under a self-generated atmosphere was less reproducible. Acetogenic metabolism, inferred from a rise in acetate levels and no increase in hydrogen yield, was frequently seen in these continuous experiments. The development of a propionate-producing culture was inferred less frequently from a raised propionate level and a decreased hydrogen yield. Both types of hydrogen removing metabolism were apparent at both pH 5.2 and pH 4.5. Reduction of the

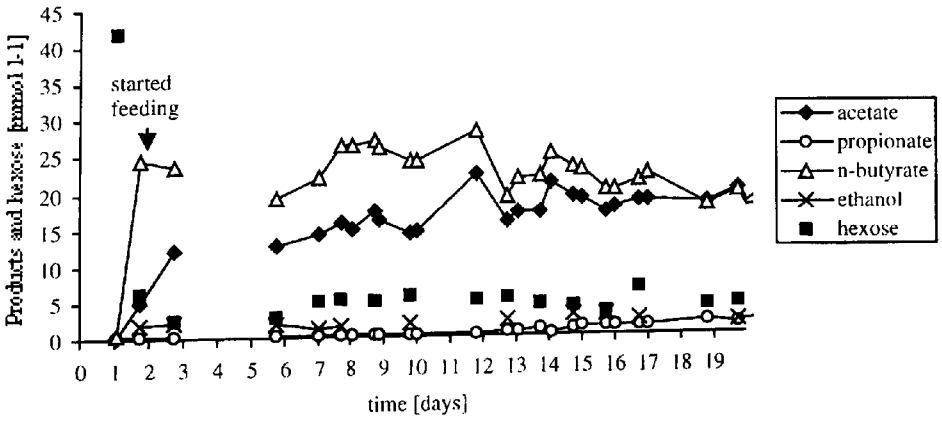


**Figure 9.** Total hexose and product concentrations during experiment 5.

HRT to 12 hours decreased propionate levels and improved hydrogen yield without affecting starch removal efficiency. Sparging with nitrogen gave more stable operation and increased hydrogen yields. An average over a 5-d period of 1.26 mol hydrogen $mol^{-1}$ hexose consumed was the highest obtained without sparging, while an average of 1.87 mol $mol^{-1}$ hexose consumed was obtained over an 18-d period with sparging.

The authors wish to thank Crespel & Deiters GmbH & Co. KG for supply of the starch.

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International Journal of Hydrogen Energy 30 (2005) 471–483
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# Continuous fermentative hydrogen production from sucrose and sugarbeet

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Received 1 December 2003; received in revised form 9 March 2004; accepted 8 April 2004

Available online 15 June 2004

**Abstract**

To produce hydrogen by fermentation of biomass, a continuous process using a non-sterile substrate with a readily available mixed microflora is desirable. This work investigates a simple batch start-up procedure at pH 5.2 and 32°C, using anaerobically digested sewage sludge, and continuous hydrogen production from refined sucrose, pulped sugarbeet and a water extract of sugarbeet. Without heat treating the sludge, and with initial nitrogen sparging, a hydrogen producing culture was established within 5 days and remained stable during two experiments of 45 and 32 days duration. At 14–15 h retention time (16 kg total sugar $m^{-3}$ $d^{-1}$ organic loading rate) hydrogen yields for refined sucrose and pulped sugarbeet were, respectively, $1.0 \pm 0.1$ and $0.9 \pm 0.2$ mol/mol hexose converted. With nitrogen sparging hydrogen yields were $1.7 \pm 0.2$–$1.9 \pm 0.2$ and $1.7 \pm 0.2$ mol/mol hexose converted for refined sucrose and water extract of sugarbeet, respectively. Increasing ethanol concentration during operation on sugarbeet, and in some cases a higher redox potential ($> -150$ mV), correlated with lower hydrogen yield.


*Keywords:* Hydrogen production; Fermentation; Sugarbeet; Redox potential; Sparging

## 1. Introduction

A process in which hydrogen was produced fermentatively from renewable biomass would encourage agricultural diversification and yield a carbon free fuel from a $CO_2$ neutral process, thus aiding atmospheric $CO_2$ reduction. The UK Department for Environment, Food and Rural Affairs has included sugarbeet (*Beta vulgaris*) in a list of energy crops (http://www.defra.gov.uk/farm/acu/energy/energy.htm). In comparison to lignocellulose energy crops, which may be more suited for hydrogen production by gasification or pyrolysis, sugarbeet offers several advantages. It is grown in annual rotation, thus generating a crop every year from planting, already commonly present in European crop rotation, and due to its high water and sucrose content is well-established as a substrate for fermentation, e.g. to bioethanol. Sugarbeet grown in the UK currently yields around 54 t $ha^{-1}$ (http://www.ienica.net/crops/sugarbeet.htm) with a water extractable sucrose content of 170 g $kg^{-1}$ wet beet (http://www.britishsugar.co.uk/bsweb/sfi/agricind/crop.htm). Dry mass of the pulp after extraction is reported to be mostly cellulose, hemicellulose and pectin, with a small amount of lignin (http://www.hdlgn-hessen.de/landwirtschaft/tierproduktion/rinder/fuetterung/press_1.htm) and variable nutrient content [1,2]. Currently sugarbeet growth is restricted to areas within delivery distance of a sugar refinery. If used as an energy crop with on-site fermentation facilities, sugarbeet could be more widely grown.

Sucrose can in theory be converted directly to hydrogen by anaerobic bacteria via fermentative butyrate/acetate

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0360-3199/$ 30.00 
doi:10.1016/j.ijhydene.2004.04.003

metabolism (Eqs. (1) and (2)), giving yields of 2–4 mol hydrogen per mol hexose converted [3]

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2)$$

Both facultative and obligate anaerobes are known to be able to convert sucrose to hydrogen. Hydrogen yields of 1.25 mol/mol hexose are reported from continuous operation with the facultative anaerobe *Enterobacter aerogenes* [4], but higher yields are expected from obligate anaerobes such as clostridia. Analysis of the microbial community of a hydrogen producing granular sludge yielding 2.2 mol hydrogen per mol hexose from sucrose [5] showed that at least 65% of species belonged to the obligate anaerobic spore-forming genus Clostridium.

The production of hydrogen in continuous fermentation utilising a non-sterile substrate with a readily available mixed microflora would be commercially desirable. Anaerobic sewage sludge from a sedimentation tank [6,7], anaerobic digester sludge [8], aerobic activated sludge [9,10], soil and compost [11] have been used as inoculum.

Fermentative hydrogen production directly from sugarbeet has not been investigated to date. Hydrogen production has been reported from pure sucrose [8,12] from sugary wastewater [10,13] and dilute molasses [4,14]. Most continuous experiments with sucrose focused on mesophilic cultures, operating at 26°C [7] to 37°C [8]. However, thermophilic continuous hydrogen production from a sugary wastewater has also been demonstrated [10].

To select against hydrogen consuming methanogens in mixed cultures on sucrose, reactors were operated at pH 5.5 [7,8,11]. Short hydraulic retention times (HRT) of less than 13.3 h [6,9] were also found to select against methanogens on sucrose at pH 6.7. Anaerobic sludge inoculum has been heat-treated at 100°C for 15 min [8], and soil inoculum baked at 104°C for 2 h [11] to select for clostridia and against non-spore forming hydrogen consumers such as methanogens. In the experiments with heat-treated anaerobic sludge inoculum continuous gas production with >50% hydrogen started within 24 h [8]. However, heat treatment may also have a disadvantage for hydrogen production, in that it selects against non-spore forming facultative anaerobes. In work with pure clostridial cultures addition of a reducing agent such as cysteine to the medium is common practice to produce a reducing environment for growth [15], since most clostridial species cannot tolerate oxygen. By consuming any traces of oxygen in a reactor and thus providing a reduced environment, facultative anaerobes may therefore promote hydrogen production by obligate anaerobes at lower cost than chemical addition. Yokoi et al. [16], for example, showed that addition of the facultative anaerobe *E. aerogenes* to a pure culture of *Clostridium butyricum* growing on starch not only enabled *C. butyricum*, which required a reducing agent for growth, to convert the starch to hydrogen, but also achieved hydrogen yields of 2 mol/ mol glucose without any reducing agent.

However, to date in studies where the natural inoculum was not heat pre-treated, start-up on sucrose is reported to be a lengthy procedure. Chen and Lin [6] used a start-up procedure which took 56 days, during which the culture was fed semi-continuously with a decreasing HRT from 20 to 2 days, and the produced gas contained only 9% hydrogen. Fang and Liu [7] increased the sucrose concentration stepwise from 2 to 12.15 g $l^{-1}$ over 20 days at start-up. Lin and Jo [9] decreased the retention time in continuous operation from 5 days to 12 h in 4 steps, each taking 20 days. Ueno et al. [10] report that their thermophilic continuous culture on sugary wastewater was initially operated at 2 d HRT for 26 days, during which gas evolution was less than 1 ml $min^{-1}$ $l^{-1}$.

Hydrogen production is thermodynamically less favourable as the partial pressure of hydrogen increases. A 68% increase in the hydrogen yield from glucose was obtained by reducing hydrogen partial pressure through sparging with nitrogen [17]. Using heat-treated anaerobic sludge inoculum and a co-product of the wheat starch industry Hussy et al. [18] have shown that nitrogen sparging increased the hydrogen yield from 1.3 to 1.9 mol/mol hexose consumed.

The experiments here examined the feasibility of continuous hydrogen production from sucrose and sugarbeet with and without nitrogen sparging, using a simple batch start-up procedure with non-heat treated sludge inoculum. Redox potential, fermentation end products, liquid phase mass balance and hydrogen yields during continuous operation were determined.

## 2. Materials and methods

### 2.1. Experiments

In three experiments following a batch start-up period on refined sucrose the system was converted to continuous operation on the same substrate. The reactors were operated at pH 5.2, 32°C and 15 or 14.2 h HRT. Details of the experiments are shown in Table 1. In experiments 2 and 3, the feed was changed to sugarbeet water extract with or without water-extracted pulp during periods of continuous operation as shown in Table 1. Experiment 2 continued for 44 days after start-up, and experiment 3 for 31 days. Both experiments were terminated only due to time constraints.

### 2.2. Substrate

The carbohydrate substrate used in these experiments was refined sucrose or water extract from pulped sugarbeet, giving an organic loading rate (OLR) of approximately 16 kg $m^{-3}d^{-1}$. In experiment 3, as shown in Table 1, water extracted pulp was added to the water extract. Sugarbeet, the principle substrate under investigation, was substituted

Table 1
Operating parameters and duration of experiments 1–3

| Exp. | Batch start-up | | Continuous operation | | |
|---|---|---|---|---|---|
| | Duration of batch start-up [days] | Sparging [yes/no] | Duration of cont. feeding [days from start-up] | Sparging [yes/no] | Substrate |
| 1 | 3.1 | No | 3.1–9.9 | No | Sucrose |
| 2 | 1.0 | Yes | 1.0–17.0 | Yes | Sucrose |
| | | | 17.0–34.1, 38.2–41.0 | No | Sucrose |
| | | | 34.1–38.2, 41.0–45.0 | No | Sugarbeet water extract and water extracted pulp |
| 3 | 1.0 | Yes | 1.0–11.0, 18.1–24.9 | Yes | Sucrose |
| | | | 11.0–18.1, 24.9–31.9 | Yes | Sugarbeet water extract |

by sucrose during batch start-up and the beginning of continuous operation because of the technical complications of continuous operation on laboratory scale with fibrous substrate, such as blocking of feed tubes and the overflow pipe. The refined sucrose was food grade granulated sugar from Tate & Lyle (London, UK) or Silver Spoon (Peterborough, UK), dissolved in hot tap water as 200 g $l^{-1}$. This solution and the sugarbeet water extract were stored at 4°C and diluted to 55.6 mmol $l^{-1}$ (10 g $l^{-1}$) at the point of entry to the reactor.

Whole fresh sugarbeet was supplied by ADAS (Ely, UK). Within 3 days of arrival the beet was peeled and stored at −20°C for up to 8 months. Before use the beet was defrosted and pulped with equal amounts (1:1 w/w) tap water in a domestic blender (Moulinex, Birmingham, UK). Pumps to deliver the pulp are not available at laboratory scale [19], so water extract was used for continuous feeding. Pulped beet (1.5–2 kg) was squeezed through a 15 μm mesh muslin. The residual pulp was twice more re-suspended in cold tap water (1:1 w/w of the original beet weight) and filtered through the cloth. The extract from the 3 steps was combined and is referred to as "water extract". Water extract and water extracted pulp were stored separately in aliquots at −20°C. The water extract was defrosted immediately before use and fed continuously through a peristaltic pump (505S from Watson Marlow, Falmouth, UK; inner diameter of tubing 1.85 mm). For days 34–38 and 41–45 of experiment 2 (see Table 1) the residual water extracted pulp was mixed 1:3 (w/v) with water and injected daily manually through the sampling port with a plastic syringe in the original water extract:water extracted pulp ratio obtained from the beet. The addition of 100 ml pulp in water twice daily, in addition to continuous feeding with sugarbeet water extract, lowered the HRT from 15.0 to 14.2 h. In experiment 3, to reduce bacterial growth in the feed tube in warm weather, the water extract was acidified to pH 2.9 with 1 M HCl during days 25–27. Mineral nutrients were added to all carbohydrate sources as described in Hussy et al. [18].

### 2.3. Inoculum and start-up

The inoculum, anaerobic digester sludge from a sewage biosolids mesophilic digester (Cog Moors Sewage Treatment Works, Cardiff, UK), sieved through a 1.18 mm mesh, was stored in a sealed container at room temperature. For experiments 1, sludge had been stored for 17 weeks before start-up. Experiments 2 and 3 were started up with batches of sludge which were stored for 21 and 7 days, respectively. The sludge was added to give 3 g $l^{-1}$ TS in the reactor containing growth medium. Continuous operation was started once significant hydrogen production had occurred.

### 2.4. Reactor set-up

Two anaerobic CSTR reactors with working volumes of 9.0 (experiment 1) and 2.3 l (experiments 2 and 3) were used. Each reactor was stirred with an adjustable stirrer (Heidolph Instruments, Schwabach, Germany) at 100 rpm. Temperature was controlled by a Grant flow heater (Cambridge, UK) to 32°C through a water jacket. The pH value was kept constant between 5.2 and 5.3 by automatic titration with a Watson Marlow (Falmouth, UK) 505U/RL pump connected to a Mettler Toledo (Urdorf, Switzerland) pH controller, using 1 M NaOH. All ingredients were pumped into the reactor by Watson Marlow 505U/RL or 505S pumps. The liquid level in the reactor was controlled by the position of an overflow pipe. For sparging a shortened sinter-stick with 10–16 μm pore size (Fisher Scientific, Loughborough, UK) attached to the centre of the 2.3 l reactor base was connected to a GC grade nitrogen cylinder from Messer (Reigate, UK). The sparging rate ranged from 40 to 70 ml $min^{-1}$. It was measured with a volumetric flow metre from Cole Parmer (Vernon Hills, Illinois, USA). During sparging the hydrogen and carbon dioxide production rates were directly calculated from the total outgoing gas flow and its hydrogen and carbon dioxide content, respectively. The

average total gas flow rate during the 30 min before gas sampling for hydrogen analysis was used for calculation of the hydrogen production rate.

The exiting gas flow rate, carbon dioxide content of the exiting gas, redox potential and culture pH were monitored continuously. Data was logged every 5 min (as the average of data recorded every 30 s) to a Viglen Pentium III computer fitted with a Labview data acquisition system. The exiting gas flow rate was measured with a volumetric flow meter from Agilent (Placerville, USA) or an Alexander Wright low flow gas meter LM300 operating on a principle developed in this laboratory [20]. The percentage carbon dioxide in the produced gas was measured with a meter fitted with an infrared gas card from Edinburgh Sensors Ltd. (Livingstone, UK). The redox potential was measured with a combination redox electrode type Pt4805-DXK-S8/120 from Mettler Toledo (Leicester, UK).

### 2.5. Off-line analysis

Gas samples were taken after the effluent gas passed through a silica moisture trap. Fluid samples were taken from a port near the bottom of the reactor. The hydrogen content of the produced gas was determined daily off-line by the method of Mizuno et al. [17]. VFAs were determined daily off-line by the method of Cruwys et al. [21]. Acetone, ethanol and butanol were determined daily by gas chromatography by the method of Hussy et al. [18]. Lactate and formate were analysed in experiments 2 and 3 by liquid chromatography (HPLC (Dionex, Camberley, UK) fitted with an IONPAC ICE-AS6 column for organic acids, anion-ICE micromembrane suppressor (AMMS-ICE II) and conductivity detector (CD20), 0.4 mM heptafluorobutyric acid eluent at 1.0 ml $min^{-1}$). Total solids (TS) of the sludge inoculum and the sugarbeet were determined by the standard method [22], as were volatile suspended solids (VSS) of reactor samples daily from day 10 of experiment 2 and throughout experiment 3. Inorganic carbon (IC) and total organic carbon (TOC) were determined with a DC-190 high temperature TOC analyser (Rosemount Dohrmann, Santa Clara, US). For determination of filtered TOC the sample was passed through a Whatman GF/C filter (1.2 μm pore size) before analysis. Total sugars were quantified with the phenol–sulphuric acid assay [23].

For mass balance calculations on days 13.0–17.0, 27.5–31.5 and 36.0–38.5 in experiment 2 and days 28.0–32.0 in experiment 3 all components were converted to mg C $l^{-1}$ reactor. When feeding with sucrose the conversion for VSS to mg C was based on the assumption that all VSS are microbial biomass and contain 45.5% carbon [24]. When feeding sugarbeet water extract, mass balance was only calculated for filtered TOC, since the particles in the water extract would be included in the VSS.

## 3. Results and discussion

### 3.1. Sugar content of sugarbeet and its components

Analysis of randomly selected chips of frozen beet showed that the peeled beets after storage at −20°C contained approximately 22–27% TS and 180–210 g total sugar $kg^{-1}$ wet sugarbeet, in comparison to the average sucrose concentration of approximately 170 g $kg^{-1}$ for commercially refined British sugarbeet (http://www.britishsugar.co.uk/bsweb/sfi/agricind/crop.htm).

After separation of the water extract and water-extracted pulp for continuous feeding in experiments 2 and 3, the batches of water extract were found to contain 38–60 g total sugar $l^{-1}$ and the residual pulp 5.5–7.6 g total sugar $kg^{-1}$ wet weight. Therefore over 98.5% of sugar was extracted into water. In experiment 2 the daily total sugar supplied with the pulp during days 34–38 and 41–45 was approximately 1% of the total sugar supplied with the water extract.

### 3.2. Experiment 1

Experiment 1 investigated continuous hydrogen production from refined sucrose without sparging. Fig. 1a shows that in experiment 1, for which sludge inoculum had been stored for 17 weeks, hydrogen production started after a lag phase of 2.5 days (60 h). The redox potential had decreased to below −200 mV before the onset of gas production, indicating microbial activity that did not involve measurable gas production but consumed oxygen. During days 2.5–3.1 gas production increased from 0.1 to 3 ml $min^{-1}$ $l^{-1}$, containing 60–70% hydrogen (Fig. 1a). Butyrate was the main product (Fig. 1b) during batch start-up, reaching a concentration of 11.8 mmol $l^{-1}$ (1040 mg $l^{-1}$) on day 3.2. Significant amounts of acetate (8.1 mmol $l^{-1}$ or 490 mg $l^{-1}$) and small amounts of ethanol (1.2 mmol $l^{-1}$ or 60 mg $l^{-1}$) were also produced by day 3.2.

Continuous operation commenced on day 3.1 by feeding of refined sucrose. During days 3.8–7.1 the gas production varied from 2 to 4 ml $min^{-1}$ $l^{-1}$ in oscillations (Fig. 1a). Butyrate continued to be the main product, its concentration increasing to a maximum of 26.7 mmol $l^{-1}$ (2350 mg $l^{-1}$) on day 7.1, whilst the acetate concentration increased to a maximum of only 13.0 mmol $l^{-1}$ (780 mg $l^{-1}$) on day 4.2. Over 95% of total sugar was converted on day 7.1. Throughout continuous operation the redox potential increased gradually (Fig. 1a) from −350 mV on day 3.8 to −280 mV on day 5.8, but steeply thereafter, reaching 0 mV on day 7.5 and +160 mV on day 9.2. From day 7.1 clear signs of washout appeared. During days 7.1–9.2 the residual total sugar concentration increased from 0.4 to 8.1 g $l^{-1}$, whilst butyrate and acetate concentrations decreased from 26.7 and 8.2 mmol $l^{-1}$ (2350 and 490 mg $l^{-1}$) to 6.7 and 3.2 mmol $l^{-1}$ (590 and 190 mg $l^{-1}$) respectively (Fig. 1b). The gas production decreased gradually and ceased on day 8.8 (Fig. 1a).

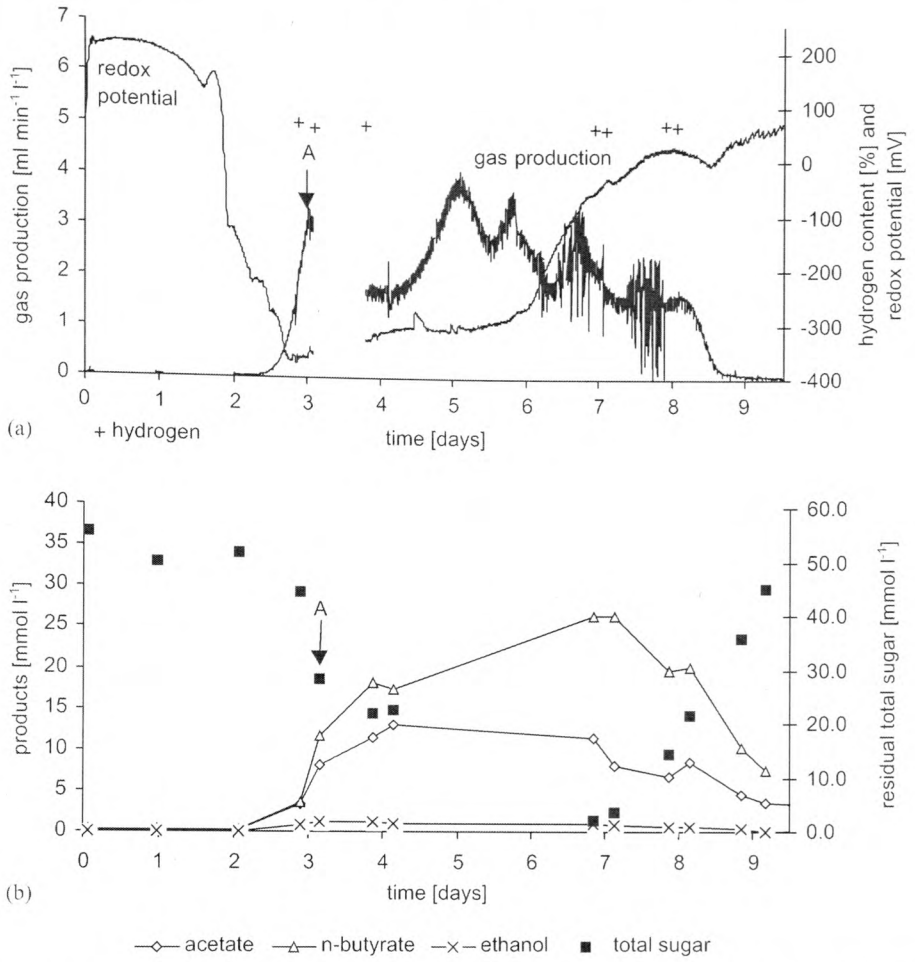


Fig. 1. Experiment 1. Batch start-up and continuous operation on sucrose (no nitrogen sparging). Arrow A: continuous operation started. (a) Gas production [ml $min^{-1}$ $l^{-1}$], $H_2$ [%] and redox potential [mV]. (b) Residual total sugar and products [mmol $l^{-1}$].

Fig. 1a shows that the redox potential is negatively related to the rate of gas production. Butyrate-acetate and hydrogen producing metabolism is reported to be associated with a redox potential of around −300 mV for a mixed culture [24], which is in accordance with the redox potential during days 2.5–5.8 of experiment 1, but means that after day 5.8 the redox potential was possibly too high for hydrogen producers. The strong increase in redox potential from day 5.8, one day before total sugar conversion decreased, suggests that redox potential may be used as an early indicator for deterioration of a hydrogen producing culture. Since there were no technical problems or changes in the dominant culture metabolism to account for washout (propionic acid, acetone and butanol concentrations were $<50$ mg $l^{-1}$ and the butyrate/acetate and hydrogen producing metabolism (as in Eqs. (1) and (2)) was dominant throughout), it is possible that the evolving culture was not able to consume the oxygen added with feed and water during continuous operation. This would cause an increase in redox potential and sporulation of oxygen sensitive clostridial species.

### 3.3. Experiment 2

Experiment 2 investigated whether sparging with nitrogen and use of fresher inoculum would allow the culture to maintain a low redox potential, thus preventing washout of oxygen sensitive clostridia. Sparging was used throughout batch start-up and continuous operation on sucrose until day 17.0, when sparging was stopped so that hydrogen yields for refined sucrose in sparging and non-sparging conditions could be compared. From days 34.1–38.2 and 41.0–45.0 sugarbeet water extract and water extracted pulp were used as substrate so that hydrogen yields in non-sparging conditions could be compared with those for refined sucrose.

#### 3.3.1. Operation on refined sucrose with sparging

Hydrogen and carbon dioxide production started within less than 16 h (Fig. 2a). This lag phase was considerably shorter than that of 60 h in experiment 1 (Fig. 1a) and approached the lag phase of 14 h reported by Sung et al. [8] from heat-treated sludge inoculum on sucrose at pH 5.5.

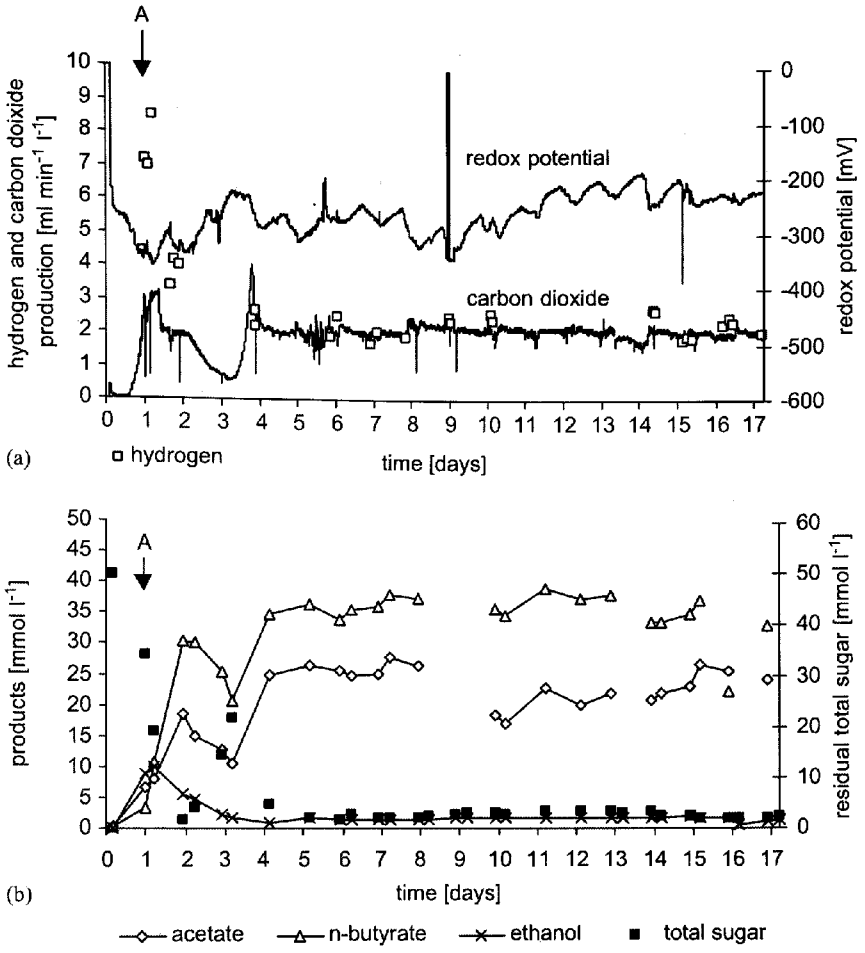


Fig. 2. Experiment 2, days 1–17. Batch start-up and continuous operation on sucrose with nitrogen sparging. Arrow A: continuous operation started. (a) Hydrogen and carbon dioxide [ml $min^{-1}$ $l^{-1}$] and redox potential [mV]. (b) Residual total sugar and products [mmol $l^{-1}$].

This shorter lag phase could be due to use of fresh inoculum stored for only 3 weeks (compared to storage for 17 weeks in experiment 1) or sparging with nitrogen in experiment 2. It suggests that heat treatment of the sludge inoculum may not necessarily shorten the start-up procedure. As in experiment 1, the redox potential had decreased to −260 mV before gas production started, and decreased further to −330 mV by the time continuous operation commenced (Fig. 2a). The decrease in redox potential to −260 mV happened within 6 h in experiment 2 compared to 43 h in experiment 1, which may indicate more activity by facultative anaerobes removing oxygen in the fresher sludge, or effects of nitrogen sparging.

Fig. 2a shows hydrogen and carbon dioxide production rates [ml $min^{-1}$ $l^{-1}$] in sparging conditions calculated from continuous measurements of total gas and on- or offline measurement of its carbon dioxide or hydrogen content, respectively. Fig. 2a shows that in experiment 2 hydrogen production increased to over 7 ml $min^{-1}$ $l^{-1}$ within the first 24 h, giving with carbon dioxide a gas production of over 10 ml $min^{-1}$ $l^{-1}$, compared to 3 ml $min^{-1}$ $l^{-1}$ at the start of continuous operation (day 3.1) of experiment 1. In contrast to experiment 1, ethanol was the main product after 24 h with a concentration of 8.9 mmol $l^{-1}$ (410 mg $l^{-1}$), when continuous operation was started (Fig. 2b). Butyrate and acetate concentrations after 24 h were 3.2 and 6.6 mmol $l^{-1}$ (280 and 390 mg $l^{-1}$).

With start of continuous operation butyrate production became dominant, reaching a concentration of 30.4 mmol $l^{-1}$ (2670 mg $l^{-1}$) on day 1.9 (Fig. 2b). Acetate concentration increased to 18.5 mmol $l^{-1}$ (1110 mg $l^{-1}$) on day 1.9, whilst ethanol concentration decreased gradually to < 1.7 mmol $l^{-1}$ (< 80 mg $l^{-1}$) on day 3.2, where it remained during continuous operation on sucrose. After day 2.2 (after 2 retention times) signs of washout (as at the end of experiment 1) appeared, with butyrate and acetate concentrations decreasing to a minimum of 20.7 and 10.4 mmol $l^{-1}$ (1820 and 630 mg $l^{-1}$) respectively

(Fig. 2b), on day 3.2, coinciding with an increase in redox potential from −330 mV on day 2.2 to −230 mV on day 3.2, and a decrease in carbon dioxide production from 2 ml $min^{-1}$ $l^{-1}$ on day 2 to 0.5 ml $min^{-1}$ $l^{-1}$ on day 3 (Fig. 2a). At the same time the residual total sugar concentration increased from 1.7 mmol $l^{-1}$ (0.3 g $l^{-1}$) on day 1.9 to 21.7 mmol $l^{-1}$ (3.9 g $l^{-1}$) on day 3.2 (Fig. 2b). Unlike on day 8 of experiment 1 however, washout was reversed after day 3.2.

During days 5–17 the redox potential, which had increased to −230 mV during the washout on day 3, varied strongly from −340 to −200 mV, with no clear relation to hydrogen production. Hydrogen and acid production were steady with an average hydrogen yield of 1.9 ± 0.2 mol/mol hexose converted. Butyrate was the main product with a concentration of 35.2 ± 3.8 mmol $l^{-1}$ (3100 ± 330 mg $l^{-1}$), and the average acetate concentration was 23.6 ± 0.3 mmol $l^{-1}$ (1410 ± 190 mg $l^{-1}$). No acetate or lactate was produced, and ethanol, propionate and formate concentration remained below 100 mg $l^{-1}$. Over 95% of substrate was consumed. A mass balance for days 13.0–17.0 (Table 2) shows that 100% of the TOC in the liquid phase of the reactor was accounted for, confirming that there were no other significant fermentation products.

### 3.3.2. Operation on refined sucrose without sparging

On day 17.0 of experiment 2 the sparging gas was turned off to investigate if this would cause a decrease in hydrogen yield or even washout. Hydrogen production decreased from 2.3 ml $min^{-1}$ $l^{-1}$ on day 17 (total exiting gas flow of 58.6 ml $min^{-1}$, containing 9% hydrogen) to 1.1 ml $min^{-1}$ $l^{-1}$ on day 21 (gas production of 4.7 ml $min^{-1}$, containing 55% hydrogen), but varied little during days 21–32 (Fig. 3a). The average daily gas production during days 21–32 was 2.1 ± 0.4 ml $min^{-1}$ $l^{-1}$ with a hydrogen content of 54 ± 2%. This gives an average daily yield of 1.0 ± 0.1 compared to 1.9 ± 0.2 mol hydrogen per mol hexose converted during days 5–17 with nitrogen sparging (Table 3). Sparging therefore increased the hydrogen yield by 90% in this experiment. During days 21–24 the non-gaseous product concentrations were very similar to those during nitrogen sparging: the average butyrate and acetate concentrations were 35.6 ± 0.9 mmol $l^{-1}$ (3130 ± 80 mg $l^{-1}$) and 26.3 ± 1.1 mmol $l^{-1}$ (1580 ± 70 mg $l^{-1}$) respectively, and >95% of substrate was consumed (Fig. 3b).

Similarly, Mizuno et al. [17] reported from a mixed enrichment culture on glucose that hydrogen yields were increased by 68% through sparging, whilst the concentration

Table 2
Mass balance [mg C $l^{-1}$] for 4 periods of experiments 2 and 3

| Experiment | 2 | 2 | 2 | 3 |
|---|---|---|---|---|
| Days | 13.0–17.0 | 27.5–31.5 | 36.0–38.5 | 28.0–32.0 |
| TOC of liquid phase | 3669 | 3288 | 3383 | 2637 |
| VSS[a] | 1185 | 891 | | |
| TOC filtered | 2519 | 2406 | 1967 | 2028 |
| Acetate | 570 | 555 | 665 | 519 |
| Propionate | 26 | 29 | 26 | 5 |
| *i*-Butyrate | 17 | 30 | 27 | 27 |
| *n*-Butyrate | 1634 | 1457 | 983 | 1077 |
| *i*-Valerate | 5 | 6 | 6 | 11 |
| *n*-Valerate | 0 | 0 | 0 | 0 |
| Acetone | 0 | 0 | 0 | 0 |
| Ethanol | 42 | 27 | 89 | 231 |
| Butanol | 17 | 9 | 9 | 42 |
| Formate | 3 | 11 | 5 | 0 |
| Lactate | 0 | 0 | 0 | 2 |
| Residual sucrose | 176 | 131 | 129 | 129 |
| Σ filtered carbon in liquid phase | 2486 | 2250 | 1935 | 2042 |
| % filtered TOC of liquid phase accounted for | 98.7 | 93.5 | 98.4 | 100.7 |
| Σ total carbon in liquid phase (includes VSS) | 3671 | 3141 | | |
| % total TOC of liquid phase accounted for | 100.1 | 95.6 | | |

[a] Based on assumption that biomass contains 45.5% carbon [22].

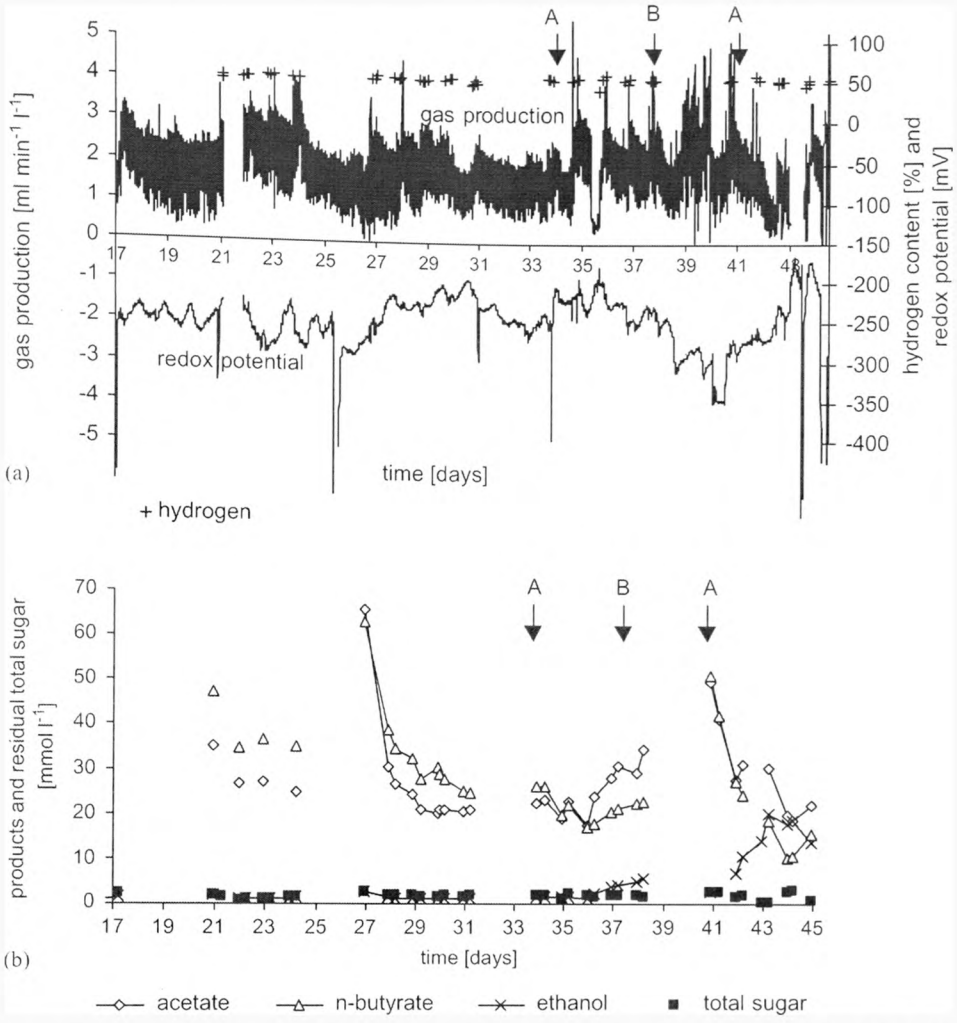


Fig. 3. Experiment 2, days 17–45. Continuous operation on sucrose (days 17–34 and 38–41) and sugarbeet (days 34–38 and 41–45) without sparging. Arrow A: operation on sugarbeet started. Arrow B: operation on sucrose started (a) Gas production [ml $min^{-1}$ $l^{-1}$], $H_2$ [%] and redox potential [mV]. (b) Residual total sugar and products [mmol $l^{-1}$].

Table 3
Yields [mol hydrogen per mol hexose converted] from sucrose and sugarbeet with and without sparging

| Substrate | Sparging | Exp. | Days | Yield |
|---|---|---|---|---|
| Sucrose | Yes | 2 | 5–17 | 1.9 ± 0.2 |
| Sucrose | Yes | 3 | 5–11 | 1.7 ± 0.2 |
| Sugarbeet juice | Yes | 3 | 28–32 | 1.7 ± 0.2 |
| Sucrose | No | 2 | 21–32 | 1.0 ± 0.1 |
| Sugarbeet juice and pulp | No | 2 | 34–38 and 41–45 | 0.9 ± 0.2 |

of butyrate, which was also the main product, was increased by just 10% and that of acetate was not affected at all. Since in experiment 2 over 95% of the substrate was converted with or without sparging, an increase in the production of hydrogen would be accompanied by increased acetate production at the cost of other products or biomass (Eqs. (1) and (2)), but this was not observed. As shown in Table 4 the theoretical hydrogen yield associated with the butyrate and acetate production (calculated from Eqs. (1) and (2)) during times of sparging (days 5–17) and non-sparging (days

Table 4
Actual acetate, butyrate and hydrogen yields and theoretical hydrogen yield [mol product $mol^{-1}$ hexose converted] associated with acetate and butyrate production in experiment 2

| | Sparging | Non-sparging |
|---|---|---|
| | Days 5-17 | Days 21–24 |
| Actual butyrate yield | 0.66 | 0.66 |
| Actual acetate yield | 0.45 | 0.48 |
| Actual hydrogen yield as calculated from total exiting gas flow and hydrogen content | 1.9 | 1.1 |
| Theoretical hydrogen yield associated with acetate and butyrate yield as in Eqs. (1) and (2) | 3.1 | 3.2 |

21–24) would be 3.1 and 3.2 mol hydrogen per mol hexose converted, respectively. The actual yields are only 61% and 34% of these theoretical values, respectively. This suggests that either a significant proportion of the acetate is produced by homoacetogens and thus associated with hydrogen consumption rather than hydrogen production, or that a significant proportion of hydrogen is dissolved and washed out with the effluent. Sparging with nitrogen may therefore have reduced this loss of produced hydrogen by 45%.

The high butyrate and acetate concentrations shown for day 27 in Fig. 3b were caused by a blockage of nutrient and water supply on day 26, which caused a 12 h increase in the HRT to 12.5 days without changing the OLR of 16 kg $m^{-3}d^{-1}$. However gas production was unaffected (Fig. 3a). Once water and nutrient supply was reinstated and the HRT returned to 15 h, butyrate and acetate concentrations decreased to 38.9 and 30.5 mmol $l^{-1}$ (3430 and 1830 mg $l^{-1}$) by day 28. A mass balance for days 27.5–31.5 (Table 2) shows that 96% of TOC in the liquid phase of the reactor were recovered, confirming that all major non-gaseous products are accounted for.

No significant difference in the redox potential between sparging and non-sparging conditions could be detected, with an average of $-253 \pm 37$ mV during days 5–17 (sparged) and $-250 \pm 36$ mV during days 18–33 (non-sparged). This indicates that nitrogen sparging does not determine the redox potential once a continuous hydrogen producing culture is established.

### 3.3.3. Operation on sugarbeet without sparging

On days 34.1–38.2 and 41.0–45.0 the substrate was changed from refined sucrose to whole sugarbeet, with refined sucrose fed days 38.2–41.0. The sugarbeet water extract was fed continuously at approximately 10 g sucrose $l^{-1}$. Additionally, water extracted pulp was injected twice daily during days 34–38 and 41–43. The residual fibre in the sugarbeet water extract settled in the feed tubing, and the fibre in the pulp repeatedly blocked the overflow pipe. These problems, which could probably be overcome in larger scale operation, together with accidental blocking of water and nutrient supply on day 41 for 12 h (as on day 26) are most likely the cause of the variability in product concentrations during days 34–45 (Fig. 3b). Ethanol production, which was negligible during operation on sucrose with and without sparging (concentrations were less than 2.7 mmol $l^{-1}$ or 130 mg $l^{-1}$), increased from day 37 to a maximum concentration of 20.4 mmol $l^{-1}$ (940 mg $l^{-1}$) on day 43. However, despite the irregular feed rate the hydrogen producing culture did not wash out and >90% of total sugar was consumed at all times. The average daily gas production rate varied from 1.8 to 2.5 ml $min^{-1}$ $l^{-1}$, containing 52–57% hydrogen. Daily average yields varied accordingly from 0.6 to 1.2 mol hydrogen per mol hexose converted, with an average of $0.9 \pm 0.2$ mol hydrogen per mol hexose converted (excluding days 38.2–41.0, when sucrose was fed). A mass balance conducted for days 36.0–38.5 shows that 98% of filtered TOC was accounted for.

### 3.3.4. Observations from experiment 2

Experiment 2 showed that a hydrogen producing culture could be established on refined sucrose substrate when fresh un-boiled sludge was used as inoculum and the culture was initially sparged with nitrogen. Yields from refined sucrose were increased by 90% in sparging (days 5–17) compared to non-sparging (days 21–32) conditions. Increases in hydrogen yield through sparging were also observed from a starch-rich substrate [18] and glucose [17], which suggests that this effect is not substrate-specific.

Experiment 2 showed further that continuous hydrogen production from pulped sugarbeet was possible, despite the technical problems encountered with feeding of fibrous substrate on laboratory scale. Similar hydrogen yields ($1.0 \pm 0.1$ and $0.9 \pm 0.2$ mol/mol hexose converted, respectively) were obtained from refined sucrose and pulped sugarbeet without sparging, indicating that hydrogen production from sugar beet is practically possible. Probably due to the disturbances caused by accumulation of fibre, the redox potential varied more strongly during operation on sugarbeet than on sucrose, but with an average of $-255 \pm 54$ mV was not significantly different to that during operation on sucrose ($-253 \pm 37$ mV with and $-250 \pm 36$ mV without sparging). On day 3 signs of washout were observed, during which the redox potential increased from $-330$ to $-230$ mV. However an increase in redox potential of similar magnitude was also observed during days 26–30 ($-290$ to $-210$ mV), when hydrogen production appeared stable. This suggests that an increase in redox potential in this range is not necessarily followed by deterioration in hydrogen production.

To confirm that the successful simple start-up procedure and continuous hydrogen production from refined sucrose in experiment 2 was repeatable, and to investigate whether

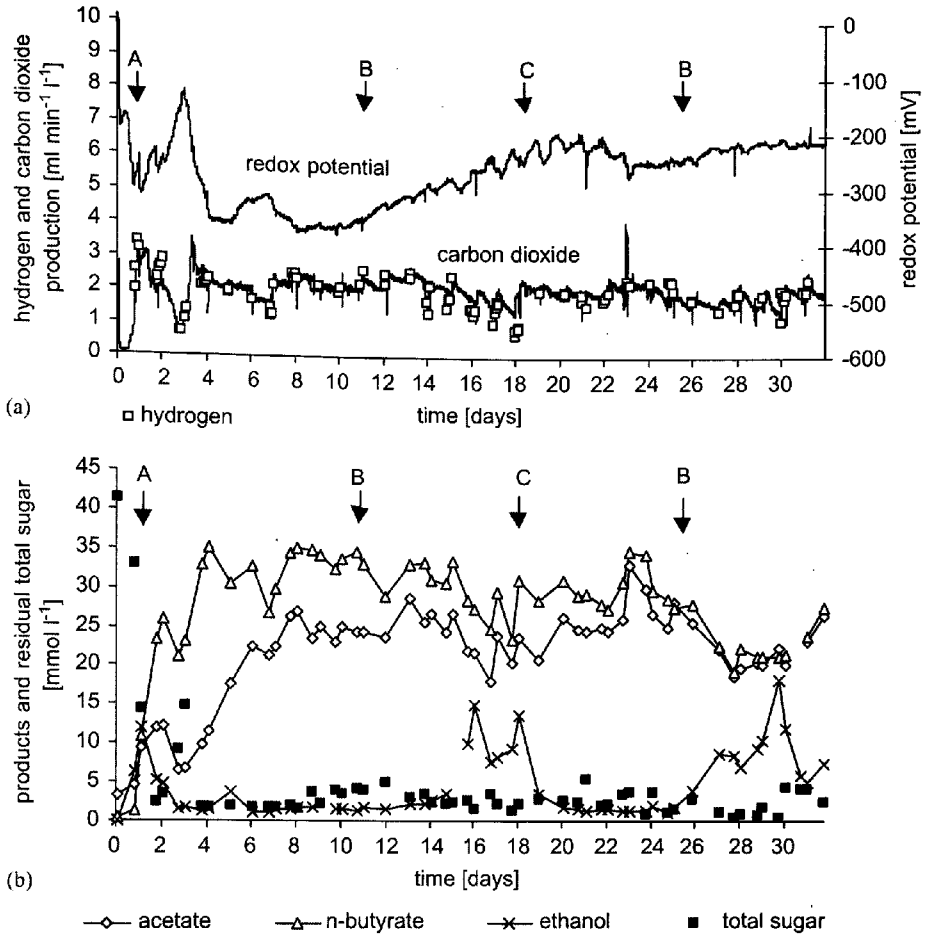


Fig. 4. Experiment 3. Continuous operation on sucrose (days 1–11 and 18–25) and sugarbeet water extract (days 11–18 and 25–32) with sparging. Arrow A: continuous operation started. Arrow B: operation on sugarbeet started. Arrow C: operation on refined sucrose started. (a) Hydrogen and carbon dioxide [ml $min^{-1}$ $l^{-1}$] and redox potential [mV]. (b) Residual total sugar and products [mmol $l^{-1}$].

hydrogen yields from sugarbeet water extract could be improved through nitrogen sparging, experiment 3 was carried out.

### 3.4. Experiment 3

#### 3.4.1. Operation on refined sucrose with sparging

The repeatability of the start-up procedure and operation with nitrogen sparging on refined sucrose (Experiment 2, days 1–17) was demonstrated in days 1–11 of experiment 3. Observations made were very similar to those in experiment 2. Hydrogen and carbon dioxide production started as in experiment 2 after approximately 16 h (Fig. 4a). The redox potential decreased to −190 mV within 5 h and further to −280 mV after 16 h (Fig. 4a). This confirms observations from experiment 2 that use of fresh inoculum and/or nitrogen sparging reduces the long lag time of 60 h observed in experiment 1. As in experiment 2, ethanol and acetate were the main products during batch start-up (day 1), but the butyrate/acetate metabolism became dominant during continuous operation (day 2; Fig. 4b).

Similar to observations made in experiment 2, signs of washout appeared on day 3, when the hydrogen production rate and butyrate and acetate concentrations showed a dip (Fig. 4a), whilst the residual total sugar concentration showed a peak (Fig. 4b). More clearly than in experiment 2 the redox potential increased steeply from −300 mV at the beginning of continuous operation on day 1 to −130 mV on day 3 (Fig. 4a). As in experiment 2 the culture recovered rapidly, with the redox potential decreasing to −360 mV on day 5. During days 5–11 the product concentrations varied little (Fig. 4b), with the exception of day 7, when the feed rate was accidentally reduced by 30% (causing reduction of the OLR by approximately 30% without significantly affecting the HRT). The average hydrogen production rate was 2.1 ± 0.3 ml $min^{-1}l^{-1}$, giving an average daily yield of 1.7 ± 0.2 mol hydrogen per mol hexose converted. This yield was slightly lower than that

of 1.9 ± 0.2 mol/mol hexose converted during days 5–17 of experiment 2 under the same condition (with nitrogen sparging and refined sucrose), but still clearly higher than the average daily yield of 1.0 ± 0.1 mol hydrogen per mol hexose converted during days 21–32 in experiment 2, when the reactor was not sparged. The average butyrate concentration was 33.9 ± 1.5 mmol $l^{-1}$ (2980 ± 130 mg $l^{-1}$) (with exception of day 7). Over 90% of substrate was converted and the redox potential remained below −300 mV.

*3.4.2. Operation on sugarbeet water extract with sparging*

On day 11 of experiment 3 the feed was changed to sugarbeet water extract. Pulp was not added because of the technical problems encountered due to accumulation of fibre in experiment 2. During days 12–18 the hydrogen producing culture appeared to deteriorate gradually. The daily average yield decreased from 2.0 on day 12 to 1.1 mol hydrogen per mol hexose converted on day 18, whilst the redox potential increased from −350 to −230 mV (Fig. 4a). Ethanol production started on day 16 (Fig. 4b), reaching a concentration of 9.5 mmol $l^{-1}$ (440 mg $l^{-1}$) on day 18. Although the daily average hydrogen yield on day 18 was still 1.1 mol/mol hexose converted, the substrate was changed back to sucrose in an attempt to halt further deterioration of the culture.

Operation on sucrose from day 18–25 caused an immediate decrease in ethanol concentration to below 2.2 mmol $l^{-1}$ (100 mg $l^{-1}$) on day 20 and prevented a further decrease in hydrogen production. The average daily hydrogen production rate was 2.0 ± 0.2 ml $min^{-1}$ $l^{-1}$ during days 19–25, giving an average yield of 1.6 ± 0.1 mol hydrogen per mol hexose converted, and the redox potential decreased slightly, to −250 mV by day 23. With exception of day 23, when sucrose inflow was accidentally increased 5–10-fold for 30 min, average butyrate and acetate concentrations during days 19–25 were 29.3 ± 1.2 and 25.0 ± 1.7 mmol $l^{-1}$ (2580 ± 107 and 1500 ± 103 mg $l^{-1}$), respectively. Since hydrogen production appeared to have stabilised by day 25, the substrate was changed back to sugarbeet water extract.

On days 26 and 27, the first 2 days after changing to sugarbeet water extract, the hydrogen producing culture appeared to deteriorate (Fig. 4b): ethanol production started again, reaching a concentration of 8.8 mmol $l^{-1}$ (400 mg $l^{-1}$) on day 27, and the daily hydrogen yield decreased to 1.25 mol/mol hexose converted. Although the ethanol concentration still increased during the following days, reaching a maximum concentration of 18.3 mmol $l^{-1}$ (840 mg $l^{-1}$) on day 29, the average daily hydrogen production rate increased from 1.7 ml $min^{-1}$ $l^{-1}$ on day 28 to 2.1 ml $min^{-1}$ $l^{-1}$ on day 32, giving an increase in hydrogen yield from 1.5–1.9 mol/mol hexose converted with an average of 1.7 ± 0.2 mol/mol hexose converted during days 28–32. A mass balance for days 28.0–32.0 shows that 99.5% of TOC in the liquid phase of the reactor was accounted for.

*3.4.3. Observations from experiments 2 and 3*

The redox potential ranged from −370 to −150 mV during continuous hydrogen production in experiments 2 and 3 (with exception of the peak of −130 mV during partial washout on day 3 of experiment 3; Fig. 4a). This was similar to redox potentials of −340 to −320 mV for a heat-treated hydrogen producing culture on sucrose [8]. In experiment 1 in comparison the redox potential had increased to −30 mV on day 7.1, when washout started. This may indicate that there exists a threshold redox value between −130 and −30 mV, which initiates sporulation in clostridia.

The hydrogen producing butyrate/acetate metabolism (Eqs. (1) and (2)) was the dominant metabolism. Butyrate was the main product throughout continuous operation on refined sucrose, with an average butyrate/acetate ratio of 1.5 mol $mol^{-1}$ during days 5–17 of experiment 2, 1.3 mol $mol^{-1}$ during days 5–11 of experiment 3 (both on sucrose with sparging) and 1.3 mol $mol^{-1}$ during days 21–24 of experiment 2 (on sucrose without sparging). During operation on sugarbeet, the butyrate/acetate ratio generally decreased with time, and was less than 1 from day 35 of experiment 2 (Fig. 3b) and approximately 1 from day 25 of experiment 3 (Fig. 4b). This change was mainly due to a stronger decrease in butyrate than acetate production. A similar gradual decrease of the butyrate/acetate ratio during continuous operation was also observed with heat-treated inoculum on sucrose [8]. In the literature successful hydrogen production from sucrose is generally associated with a butyrate/acetate ratio of greater than 1 [13,9,25]. The experiments described here confirm that to date hydrogen production from sucrose is mainly accompanied by butyrate production [12]. If the process could be biased towards acetate production as in Eq. (2), higher hydrogen yields could be expected.

Apart from the desired butyrate/acetate metabolism, ethanol production was the only other significant metabolism. It was dominant during batch start-up in experiments 2 and 3, and was also present during continuous operation on sugarbeet with and without sparging. Ethanol production (approximately 600 mg $l^{-1}$) at the start of continuous operation was also observed in hydrogen production from sucrose (fed at a concentration of 17.6 g $l^{-1}$) by heat treated inoculum [8], and thus may originate from spore-forming clostridia. During continuous operation significant ethanol production (over 1000 mg COD $l^{-1}$ from 20 g COD sucrose $l^{-1}$) was also reported at pH 6.7, 35°C and 13.3 h HRT [25], where also over 1 mol hydrogen per mol hexose was produced. Ren et al. [14] report stable continuous hydrogen production from molasses by a mixed culture with a dominant acetate/ethanol metabolism at a redox potential of around −250 mV. This metabolism, in which acetate and ethanol are produced in equal amounts, is reported to be more stable than the butyrate/acetate metabolism. In contrast, in the experiments described here, ethanol production in

continuous operation appears to coincide with periods of relatively low butyrate and hydrogen production, for example, days 43 and 44 in experiment 2 (Fig. 3a) with an average daily yield of 0.6 and 0.7 mol hydrogen per mol hexose converted, and days 16–18 in experiment 3 (Fig. 4a) with an average daily yield of 1.1–1.3 mol hydrogen per mol hexose converted.

Experiments 2 and 3 show that hydrogen yields from sugarbeet are very similar to those from refined sucrose (Table 3). With sparging, average yields of 1.9 and 1.7 mol hydrogen per mol hexose were obtained for refined sucrose during days 5–17 of experiment 2 and during days 5–11 of experiment 3, respectively, whilst an average yield of 1.7 mol hydrogen per mol hexose converted was obtained with sugarbeet water extract during days 28–32 of experiment 3. In non-sparging conditions in comparison, an average yield of 1.0 mol hydrogen per mol hexose converted was obtained for refined sucrose during days 21–32 of experiment 2, and 0.9 mol hydrogen per mol hexose converted for sugarbeet water extract and pulp during days 34–38 and 41–45 of experiment 2. Yields for operation on sugarbeet water extract (with or without added water-extracted pulp) are therefore comparable to yields obtained from pure sucrose in the experiments described here and in the literature. Sung et al. [8], for example, report yields of 0.8 mol hydrogen per mol hexose from sucrose (17.8 g $l^{-1}$) with heat-treated inoculum in semi-batch mode (pH 5.5, 37°C), Chen and Lin [25] report a yield of 1.2 mol hydrogen per mol hexose from sucrose (17.8 g $l^{-1}$) at 13.3 h HRT (max. 2.26 mol $mol^{-1}$ at 8 h HRT) in a CSTR reactor (pH 6.7, 35°C), and Lin and Jo [9] report yields of 1.3 mol hydrogen per mol hexose from sucrose (17.8 g $l^{-1}$) at 8 h HRT (pH 6.7, 35°C) in a sequencing batch reactor. Liu and Fang [13] achieved high yields of 2.2 mol hydrogen per mol hexose from a sucrose rich wastewater (sucrose content of 14.3 g $l^{-1}$), using an acidogenic granular sludge. There may be difficulties with granular sludge when the substrate contains particulate matter. However, farm scale machinery such as belt presses for separation of particulate matter (for example, in animal manure) is commercially available.

For practical application sugarbeet could be fermented directly on-site in a two-stage process consisting of a hydrogen producing reactor and a methanogenic reactor. The effluent of the hydrogen producing reactor would serve as substrate for the methanogenic stage. The effluent from the methanogenic reactor retains the original amount of nitrogen, phosphate and potassium and could be used as fertiliser. In the work reported here mineral nutrients were added to the process. The requirement for nutrient addition must be examined, but if required by the hydrogen and methane-producing processes, these nutrients would be recycled during irrigation to succeeding crops.

## 4. Conclusions

- Stable continuous hydrogen production (at 32°C, pH 5.2, 15 h HRT, 10 g $l^{-1}$ refined sucrose) was achieved rapidly within 5 days from start-up, when non-heat treated anaerobic digester sludge, stored for up to 3 weeks, was used as inoculum and the culture was initially sparged with nitrogen.
- Stable continuous hydrogen production from refined sucrose and pulped sugarbeet was achieved for a period of 45 days (70 HRTs), when the experiment was ended due to time constraints. The last 28 days of this experiment were without nitrogen sparging.
- Daily hydrogen yields during continuous operation without sparging were on average 0.9 ± 0.2 mol/mol hexose converted from sugarbeet water extract and water extracted pulp, and 1.0±0.1 mol/mol hexose converted from pure sucrose.
- Through sparging average daily hydrogen yields were improved by over 66% to 1.5 mol/mol hexose converted from sugarbeet water extract and 1.7–1.9 mol/mol hexose converted from pure sucrose.
- Ethanol production appeared to be the main competing metabolism, being particularly strong during start-up and times of irregular feed rate with sugarbeet in continuous operation.
- Mass balances for periods of continuous operation on sucrose and sugarbeet water extract with and without sparging show that 95–100% of all products in the reactor liquid were accounted for. Butyrate, acetate and ethanol were the only products of significance. Acetone, butanol, lactate, formate or propionate were not produced in concentrations above 200 mg $l^{-1}$.
- Washout of the hydrogen producing culture was found to be associated with an increase in redox potential to above −130 mV. During times of strong hydrogen production the redox potential fluctuated between −340 and −150 mV, with an average of −253 ± 37 and −250 ± 36 mV with and without sparging, respectively. An increase in redox potential in this range was not a clear indicator of deterioration of the hydrogen producing culture.

## Acknowledgements

The authors wish to thank the UK EPSRC for funding through Grant number GR/R22520/01, ADAS for supply of the sugarbeet and Louise Smok for her help with the practical work.

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